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THE ANTIVIRAL ACTIVITY OF MEPACRINE IN RELATION TO MORPHOLOGICAL CHANGES PRODUCED BY THE DRUG.

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HURST, Melvin and Peters (1952) and Hurst, Peters and Melvin (1952) described the marked therapeutic action of mepacrine* against equine encephalomyelitis and Rift Valley fever in mice. Subsequent histological studies of the livers of treated animals suffering from the latter disease revealed morphological changes consequent upon administration of the drug. The liver and Kupffer cells contained cytoplasmic particles of a kind never seen in undosed mice; these particles had a strong affinity for methylene blue in sections stained with Giemsa, and were quite remarkably persistent in time (more than 120 hours after a single oral dose) and in pathologically altered cells (persistence in necrotic fragments of cytoplasm).

At about the same time we discovered that equine encephalomyelitis in the guinea-pig was not beneficially influenced by treatment with mepacrine, and we were not able to detect basophilic particles in the liver cells. It seemed therefore that a relation might exist between the particles and the therapeutic effect; our observations on this point will be presented after we have dealt in some detail with the particles themselves.

EXPERIMENTAL.

Basophilic Particles in the Tissues of Mice Dosed with Mepacrine.

When first we observed the basophilic cytoplasmic particles, we were not aware that others had noted their presence. Later, however, we came upon the description of similar particles in rats examined by Siegel and Mushett (1944), subsequently confirmed by Fitzhugh, Nelson and Calvery (1945). It appears, also, that the particles were mentioned, though with less emphasis, by Wright and Lillie (1943), and that in some animal species they may not be clearly basophilic. In what follows we shall deal but cursorily with points already covered by these

* Mepacrine in this paper refers to mepacrine dihydrochloride dihydrate.

earlier workers and enlarge upon new observations regarding the nature and significance of the particles. We may add that dosage in our animals was never sufficiently large to evoke the drastic pathological changes described by some of the above writers, though frequent repetition of the largest dose used caused considerable loss of body weight.

Description of particles.

We have examined many livers of uninfected mice dosed with mepacrine and fixed in Zenker with acetic acid, sublimate alcohol, formol saline, formol alcohol or alcohol, chloroform acetic acid. When sections are stained with Giemsa, blue particles can be seen after all these fixatives. They can also be stained in sections prepared after freeze-drying, in liver cells stained vitally or supravitally with neutral red, in smears of tissue-homogenates, and in the mitochondrial fraction prepared by differential centrifugation of homogenates.

In the normal liver cell after freeze-drying, fixing with alcohol or other suitable fixative, and staining with Giemsa, the tigroid substance may contain a few tiny granules more deeply coloured than the remainder of the substance, but these cannot be confused with the particles under consideration which differ from them in shape, size, colour and distribution. Whereas the granules in the masses of tigroid substance are purplish in colour and rounded in shape with rather indefinite outlines, the particles in mepacrine-dosed animals take the greener blue of methylene blue, and in shape tend to be angular, irregular or fleck-like rather than rounded. They are also more variable in size. Most are of the order of a fraction of 1 μ in length, but a few may reach 1 μ or, exceptionally, even 2 μ; generally speaking, they are smaller with smaller doses of the drug. As to distribution, after a single oral dose of mepacrine (500 mg./kg.)* they often tend to occur in loosely packed clumps in the perinuclear region of the cell, from which the tigroid substance is usually absent. After repeated doses they are larger, more numerous and may occur in any part of the cytoplasm, from which much or all of the tigroid substance has disappeared (Fig. 1); the cytoplasm now shows a fine reticulation not due mainly to the presence of fat, even though fine sudanophile granules are more numerous than in the liver cells of control mice and tend to occur in the same parts of the cell as the basophilic particles. They resist decolourisation with alcohol, and are still deeply coloured in sections which have been differentiated to the point where the nuclei are mere shadows. After single or repeated doses, larger and more nearly spherical basophilic masses (1–3 μ) are present in the Kupffer cells and fine granules in a few endothelial cells of the central veins, but none in the bile ducts.

In tissues fixed and processed in conventional manner, the particles in many but not all liver cells appear larger and more rounded; they then stain more feebly (from greenish yellow to medium blue) and may easily be overlooked. Under these circumstances, also, they frequently stain only at the periphery, and thus have the appearance of minute vesicles. Routine histological measures evidently cause artefact in the particles in many of the cells; these artefacts can be avoided by freeze-drying techniques.

The particles are already present within 4 hr. of a dose of mepacrine; at this

* This and previous work was carried out with mepacrine made during the late war by Imperial Chemical Industries Ltd. The supply of this is now exhausted, and we have recently used mepacrine made by another manufacturer. The maximum tolerated dose of this mepacrine is only 400 mg./kg.

time very few liver cells are involved, though those which are contain numerous particles. At this stage the Kupffer cells are much more widely affected, supporting Siegel and Mushett's belief that the initial deposits occur in cells of the reticulo-endothelial system. By 24 hr. particles are numerous in most liver cells, and they attain a maximum at 48–72 hr. Their numbers remain stationary up to about 96 hr., and particles still persist at 14 but not at 21 days.

Distribution of particles.

In mice receiving a single dose of mepacrine the only tissues other than the liver in which we have recognised basophilic particles with certainty are muscle, brown fat and renal medullary tubules, but, as Siegel and Mushett and Fitzhugh *et al.* found, in animals dosed repeatedly they are widely distributed. Thus 48 hr. after the last of 5 doses of 500 mg./kg., given on alternate days, they are widespread in cardiac, voluntary and smooth (vascular, intestinal, etc.) muscle ; they appear as rows of granules or long bacillary forms lying between the fibrillae of voluntary muscle and as granules at the poles of the sarcolemmal nuclei, while in unstriped muscle they are usually granular. Fat, particularly brown fat, shows heavy deposits of coarse particles. In the skin they are present in fibroblasts and macrophages of the corium and in cells of the hair-roots ; they are also present in fibroblasts of the interstitium of heavily involved muscle. In the kidney they are very numerous in the loops of Henle, particularly in the ascending tubules, where the cells containing them may be degenerate and desquamating ; they occur in a smaller proportion of cells of the collecting tubules and main excretory ducts, and also in the epithelium covering the glomeruli, but not in the convoluted tubules or epithelium lining the pelvis. They are present in interstitial cells between the most heavily affected tubules. In the adrenal the heaviest deposits are in the cells of the medulla and the endothelium of the blood sinuses of this region ; a few particles occur in the most superficial cortical cells. Reticulo-endothelial cells of the spleen, thymus and lymph glands may contain particles, and less frequently the general vascular endothelium. Sometimes particles are seen in white blood cells lying in the lumen of a blood vessel and occasionally apparently free in the blood. Particles are present in macrophages in the villi of the small intestine, but not certainly in the glandular epithelium of the alimentary canal. Their occurrence in the choroid plexuses and ventricular ependyma is mentioned later. It would be difficult to identify with certainty these particles in heavily basophilic cells or cells containing a basophilic reticulum, and in fact none were recognised in the pancreatic or mucous salivary acini, or in peripheral ganglion cells. The islet cells of the pancreas, the pancreatic and salivary ducts, the epithelium of gall-bladder and urinary bladder, the bronchial and alveolar epithelium of the lung, the testicular epithelium and peripheral nerves were not observed to be affected.

After 5 doses of 150 mg./kg. in the same period of time, the particles in the liver are much finer though equally numerous. After 5 doses of 70 mg./kg. we could not certainly identify particles in Zenker-fixed material taken through paraffin. After 30 doses of 50 mg./kg. in 41 days particles were found with difficulty in scanty liver cells, although macroscopically the mice were slightly yellow. It would seem, therefore, that below a certain level of dosage basophilic material is not formed in quantity sufficient to be deposited in the liver cells.

Staining reactions, solubilities, etc.

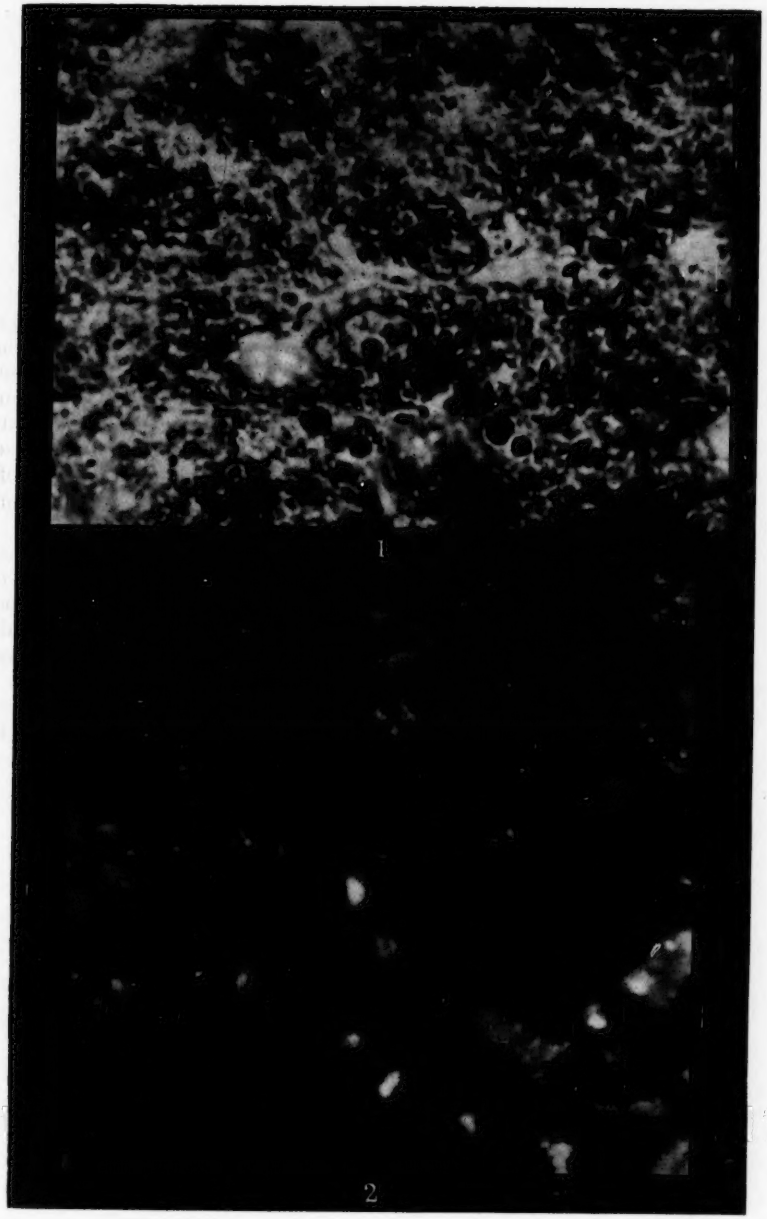
The staining of the particles with methylene blue is in no way specific. Although only the largest colour feebly with haematoxylin, and many are virtually invisible in sections stained with haematoxylin and eosin, this is not the case with other basic dyes. For instance, they are green after methyl green orange G, and they can be revealed with great clarity by Twort's stain (Twort, 1924) as deep red granules on a bright green background; the latter method is that of choice for tissue-homogenates. They stain deeply with neutral red or Janus green in liver cells examined supravitally—the staining with the latter dye is immediate—and they are also coloured in animals stained vitally with neutral red. In unstained fresh preparations the larger particles are distinctly yellowish in colour. In frozen-dried sections the particles fluoresce yellowish in ultraviolet light, though it is necessary to remove, by preliminary washing in alcohol, much other acridine causing diffuse greenish fluorescence before they can be photographed satisfactorily (Fig. 2). They do not stain in preparations coloured by phosphotungstic acid haematoxylin to demonstrate the mitochondria, so that if the latter take part in their formation they are so altered as to be unrecognisable; numerically, the particles are about one-tenth of the mitochondria, even in animals repeatedly dosed with mepacrine. They were not detected in freeze-dried material treated by the periodic acid-Schiff technique, but the presence of much glycogen hampered observation and attempts to remove it by either malt or salivary diastase led to loss of the particles (see below).

Attempts to carry out microchemical tests on the particles are complicated by their peculiar solubilities. After fixation of frozen-dried sections for 5 min. in formol saline, formol water or formol alcohol abundant particles are present; Zenker with acetic acid causes some loss. Immersion for up to 24 hr. in distilled water at room temperature, before or after 5 min. fixation in formol water or formol alcohol, has little effect on staining, but after a similar time in formol saline there is some reduction. Longer fixation in formol saline both decreases the stability to water and itself progressively reduces staining, though some particles still colour after 24 hr. in the fixative. Long sojourn in formol water is nearly innocuous. Immersion in physiological saline for 1 hr. at room temperature, before or after fixation, completely prevents basophilic staining, as do also 1 per cent sodium carbonate, hydrochloric acid or ammonium sulphate, whereas 0·3 M sucrose solution has no effect within 24 hr. Mixtures of sucrose with potassium chloride, sodium acetate or ammonium chloride are responsible for considerable loss, incomplete at 24 hr. It seems, therefore, that basophilic staining is abolished by electrolytes but not by water or sucrose at room temperature. However, water at 60° for 1 hr., after the fixatives mentioned above, abolishes staining as effectively as does saline, and water at 37° has some effect. In most instances

EXPLANATION OF PLATES.

FIG. 1.—Frozen-dried section stained with Giemsa to show the numerous basophilic particles in the cytoplasm of the liver cells and the large spherical masses in the Kupffer cells. The optical section photographed by the camera shows only a fraction of the particles visible on focussing. The mouse had received 5 doses of mepacrine in 10 days and was killed 48 hr. after the last.

FIG. 2.—Composite photograph showing in the liver cells (above) fine particles which fluoresce in ultraviolet light, and (below) larger brilliantly fluorescent masses in a tubule of the kidney.



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in which basophilic staining is no longer demonstrable, " flecks " may be seen now coloured by the counterstain; this observation suggests, of course, that the procedure employed has removed a basophilic component from a complex structure. Removal of the basophilic component is accompanied by loss of fluorescence as observed in ultraviolet light.

Disappearance of the basophilic component of the particles occurs only at certain concentrations of saline. It is not removed from frozen-dried sections even after 24 hr. by concentrations exceeding 1·1 M, and only partially at 0·55 M. At half the latter concentration removal is virtually complete, and remains so at lower concentrations down to 0·004 M, after which removal is progressively less complete. Within this range of concentrations, the staining of many normal cellular features is also lost, for example of the tigroid substance, nuclei and nucleoli, but the solubility of the basophilic substance does not tally precisely with loss of any of these (or of these with one another); the closest correlation is with the staining of the tigroid substance. Ammonium sulphate appears to be very similar to sodium chloride in its action, but magnesium chloride is less effective at concentrations down to 0·011 M. Aluminium sulphate completely ruined the staining properties (Twort's stain) of the tissues at all concentrations tested.

It follows that the effect of enzymes upon the particles can be studied only if they do not contain critical amounts of electrolyte. We obtained from America a very expensive preparation of " salt-free " crystalline ribonuclease and found that in fact it contained much electrolyte. After prolonged dialysis which freed the preparation from electrolytes, this enzyme completely removed the basophilic particles, together with much other material, from the cells; and in this case no " flecks " remained to take the counterstain. Similarly treated crystalline desoxyribonuclease left the particles intact.

No basophilic particles are formed when fixed or unfixed frozen-dried sections of liver are immersed for up to 24 hr. in 1 per cent mepacrine at room temperature or at 37°.

Particles in tissue-homogenates.

The histological observations were amplified by the bulk separation of cellular components 48 hr. after a single oral dose of mepacrine (500 mg./kg.) to mice of 20–25 g. Undosed mice were used as controls, and all were starved for 18 hr. before death. In a typical experiment the livers from about 12 mice were ground with ice-cold 0·3 M sucrose (5 ml. per 1 g. of liver pulp), and the cellular components separated by centrifugation using a method similar to that of Schneider (1948). Smears fixed with methanol and stained with Twort's stain revealed the basophilic particles only in the mitochondrial fraction; the well-defined red granules of various sizes contrasted with the rather smaller and less sharply defined mitochondria, which were coloured green. For photographic purposes the smears were fixed 15 min. in 1 per cent osmic acid in 0·3 M sucrose before being stained with Twort's stain. The particles were now coloured a deep brownish pink and the mitochondria a pale grey, thus providing sufficient contrast for photomicrography. Examination of the prints showed the particles to be mostly between 0·5 and 1 μ in diameter, and to be about one-ninth as numerous as the mitochondria. Their greater size allowed of partial concentration by differential centrifugation, but the difference in size was insufficient to permit effective separation. Preparations containing the particles were unaffected by 0·3 M ethylene glycol, but solutions of

electrolytes or treatment with water at 60° caused a loss of staining similar to that observed in sections.

Mitochondrial fractions from control and mepacrine-treated mice were fixed for 5 min. in 10 per cent neutral formalin. The flocculent precipitates, removed by centrifugation, were washed with 60 per cent ethanol and suspended for 18 hr. in Twort's stain. They were then separated and washed twice with ethanol. They were now markedly different in colour; that from untreated animals was bright green, the other brownish purple from the presence of deeply stained basophilic particles demonstrable microscopically. Suspension in benzyl alcohol extracted no colour from the former, but the precipitate from mepacrine-treated animals yielded a pink solution from which a bright green deposit could be separated by centrifugation. The solvent thus extracted neutral red from the particulate material, leaving the mitochondria stained green as in the untreated mice.

A mitochondrial fraction containing basophilic particles was washed repeatedly with methanol. The first extracts contained much yellow material, chiefly mepacrine. When the washings were no longer coloured, the residue was still pale ochre in colour and fluoresced in ultraviolet light; basophilic particles were still present. After suspension in 1 per cent sodium chloride for 18 hr. at room temperature and further washing with saline, the deposit was devoid of yellow colour and fluorescence, and the supernatant contained a fluorescent yellow substance; basophilic particles were now absent. Colourimetric observations on the methanol washings and the saline extracts suggested that the yellow material in the basophilic particles represented about 3 per cent of the total acridines present in the cells.

Macroscopic appearances.

The distribution of basophilic particles corresponds fairly closely with the macroscopic colouration of the animals. A few hours after a single oral dose of mepacrine this is most marked in the stomach, and rather later in the small intestine; thereafter colour waxes in the other organs and tissues, and by 48 hr. or so the liver, muscles, lymph nodes, salivary glands, fat, skin, etc. are the more conspicuously stained, and remain so for more than 7 days; this late colouration we will call " persistent ". An interesting point is that while the brain is faintly coloured at 4 hr., most of the nervous system does not share in this " persistent " colouration. In the later stages, however, the choroid plexuses and the lining of the ventricles are distinctly orange, an observation which recalls, without duplicating exactly, the distribution in the nervous system of an *acid* dye such as trypan blue; the colouration is associated with the presence of basophilic particles in the stroma and epithelial cells of the choroid plexus and in nearby ependymal cells lining the ventricles, whereas no particles can be recognised with certainty in other parts of the brain.

Reaction of Other Animal Species to Dosing with Mepacrine.

Macroscopic and microscopic appearances.

The *rat* on doses comparable with those tolerated by the mouse presents similar macroscopic and microscopic pictures, except that the basophilic particles in the liver cells are coarser and more conspicuous.

The *rabbit* at a dose of 250 mg./kg. behaves similarly to the rat.

The *guinea-pig* unfortunately does not tolerate mepacrine as well as does the mouse. Nevertheless, it will accept doses (whether calculated on the metabolic rate formula or on a plain weight for weight basis) within the range capable of producing " persistent " colouration of the tissues and basophilic particles in the liver cells of the mouse. We have observed neither of these phenomena in the guinea-pig.

Although within a few hours of a single dose of mepacrine (150–250 mg./kg.) there is some yellow colouration of the tissues, and slight colour may persist in the intestine for about 24 hr., general staining of the animal does not last so long; nor is appreciable colour present in animals killed 48 hr. after the last of 5 doses of 150 mg./kg. on alternate days, or in those killed 24 hr. after a total dose of 2·5 g./kg. in 28 days or 5 g./kg. in 66 days. An exception to the foregoing statement is the outer medullary zone of the kidney, which after repeated dosing is vividly orange in colour. Frozen-dried sections of liver are not clearly yellow, as in the mouse.

On microscopical examination of conventionally processed or frozen-dried tissue no basophilic particles are visible in the liver cells, muscles, fat, etc., though after repeated dosing abnormal material is present in the Kupffer cells. This material differs from that in the mouse, for when stained with Giemsa the masses appear yellow-green to grass-green rather than intense blue. In unstained sections it consists of aggregates of coarse, distinctly yellow granules, and were tiny flecks of this nature present in the cytoplasm of the liver cells, in the absence of marked basophilia they might well be invisible. However this may be, similar material to that in the Kupffer cells abounds in the cells lining the sinuses of the adrenal medulla, and coarse yellowish granules in the medullary cells themselves, though not in local nerve cells or in cells of the adrenal cortex. The epithelial cells of the choroid plexuses are heavily laden with similar coarse granules. The renal tubules, on the other hand, contain much intensely basophilic material similar to that in the mouse, mixed with some yellow-green or grass-green deposits; they are present in the Henle tubules, in adjacent connective-tissue cells, and, after repeated dosing, as heavy deposits much further down in the main collecting tubules, but short of the ducts of Bellini.

The picture in the guinea-pig is no different when drug is administered intraperitoneally instead of orally.

The *dog* frequently vomits after an oral dose of mepacrine, and may then ingest some of the rejected gastric contents; it is thus difficult to be sure of the precise amount of drug received. Forty-eight hours after the last of 6 doses of 400 mg./kg. within 14 days, four dogs showed slight yellow colouration only of the small intestine and possibly of the conjunctiva. Appearances in the Kupffer cells resembled those in the guinea-pig, but in addition coarse yellow or slightly greenish granules were present in the liver cells; in unstained sections these granules were distinctly yellow and they fluoresced in ultraviolet light. Material in the renal tubules was apparently similar, and without any marked basophilic tendencies.

Monkeys (*Macacca mulatta*) given 800 mg./kg. in 3 doses in 3 days or 1·6 g./kg. in 4 doses during a period of 20 days, and killed 48 hr. after the last dose, were only slightly yellow. The former exhibited a few basophilic granules in the liver cells but, for the most part, both here and in the Kupffer cells the granules were greenish or yellowish brown; some, but not all, reacted for iron. Similar fine granules were found in the Henle tubules of the kidney.

The recently hatched *chick* is very tolerant to mepacrine (see Hegsted, McKibbin and Stare, 1944), and the organs of the fowl, with the exception of the liver, are said to store the drug in lower concentration than do those of the rabbit (Oldham and Kelsey, 1945).

Baby chicks receiving a total of 1 g./kg. in daily doses over a period of 5 days showed very deep " persistent " colouring of liver, fat, skin, muscles, etc.; the brains were also more perceptibly coloured than in other species. At 3 weeks of age, however, the brains were not coloured; similar differences were noted in the penetration of trypan blue into the brains of recently hatched and older birds.

Microscopically, basophilic particles in the liver cells were extremely coarse, and they were also very conspicuous in the spleen, fat and muscles. In the baby chicks some of the nerve cells in the central nervous system contained basophilic particles, a finding not duplicated in other species or in the 3 week-old birds. The kidneys of the baby chicks were not examined; in those at 3 weeks of age no basophilic material was clearly visible.

It seems, therefore, that whether because of differences in the metabolism of mepacrine in various animal species, or because of the presence or absence of a " receptor " substance to which mepacrine or one of its metabolites may become attached, the macroscopic and microscopic appearances in animals of various species dosed with the drug differ considerably. It remains to describe the therapeutic activity of mepacrine in the different species.

Therapeutic Response to Mepacrine in Infection with Equine Encephalomyelitis Virus.

Differences exist between the various animal species in their susceptibility to encephalitis and death following infection with the virus of equine encephalomyelitis, and in the susceptibility within any one species of animals of different ages; in part at least these differences are related to the degree of extraneural multiplication of the virus and the titre of virus resulting in the blood stream (Hurst, 1950). For present purposes, the situation is further complicated by differing degrees of tolerance to mepacrine. Table I presents some of the results of attempted treatment of the disease in hosts other than the mouse. In all these experiments treated and untreated groups of mice responded in the usual way.

A fair proportion of fatal infections occurs in the newly weaned *rat*, in which host we obtained no evidence of a beneficial effect of preliminary dosing with mepacrine, whereas with older rats and a declining susceptibility to encephalitis we were able to demonstrate a protective effect of the drug. The former result cannot be attributed solely to the immaturity of the host since, as may be seen from Table I, in the correspondingly immature mouse we obtained beneficial results nearly as marked as those normally obtained in the adolescent animal. This observation has been repeated several times. The course of the disease in baby *chicks* appears to be adversely affected by dosing with mepacrine, while in older chicks we did not obtain fatal infections in either treated or control birds. Mortality among *guinea-pigs* is seldom appreciably diminished by treatment with mepacrine at levels of dosage which still give an obvious effect in mice; this failure occurs irrespectively of whether single or multiple doses are administered.*

* Since for infecting we normally use virus propagated in guinea-pigs, the question arose whether infections in the guinea-pig with homologous virus are more difficult to influence than are infections in the mouse with heterologous virus. Suitable experiments disposed of this possibility.

TABLE I.—*Effect of Mepacrine on Eastern Equine Encephalomyelitis in Several Animal Species.*

Host, age, etc.	Animals per group.	Dosage of mepacrine.	Infecting dilution of virus.	Deaths. Treated.	Deaths. Untreated.
Rat:					
Newly weaned, 28–33 g.	12	400 mg./kg. 24 hr. before virus	10^{-4}	9 (5·7)	6 (5·8)
	12	As above	10^{-5}	5 (6·2)	7 (5·4)
Young animals, 60–70 g.	20	350 mg./kg. 24 hr. before virus	10^{-4}	1 (7·0)	10 (7·1)
„ „ 110–120 g.	12	400 „ „ „ „ „ „	10^{-4}	0	4 (8·0)
	12	As above	10^{-5}	0	4 (8·0)
Chicken:					
Baby chicks, 40–50 g.	20	200 mg./kg. daily from 24 hr. before virus	10^{-1}	20 (4·1)	12 (4·4)
	20	As above	10^{-2}	17 (4·2)	16 (4·5)
	20	„ „	10^{-3}	19 (4·3)	10 (4·1)
Month-old chicks, 150–160 g.	20	„ „	10^{-2}	0	0
	20	„ „	10^{-4}	0	0
Guinea-pig:					
Young animals, 250–300 g.	9	(*a*) 160 mg./kg. 24 hr. before virus	10^{-2}	7 (4·9)	8 (4·6)
	9	(*b*) 40 mg./kg. *b.i.d.* from 24 hr. before virus	10^{-2}	7 (5·0)	
„ „ 250–300 g.	8	As (*a*) above	$10^{-3.5}$	5 (7·6)	4 (7·0)
	8	„ (*b*) „	$10^{-3.5}$	5 (9·6)	
„ „ 220–260 g.	9	„ (*a*) „	$10^{-4.5}$	0	3 (6·6)
	9	„ (*b*) „	$10^{-4.5}$	3 (7·0)	
„ „ 250–260 g.	20	300 mg./kg. 48 and 150 mg./kg. 4 hr. before virus	10^{-3}	12 (6·2)	13 (5·3)
Rabbit:					
Young animals, 700 g.	12	300 mg./kg. 24 hr. before and 150 mg./kg. 24 hr. after virus	10^{-2}	4 (6·1)	1 (7·0)
Mouse:					
Newly weaned, 10–12 g.	20	300 mg./kg. 24 hr. before virus	10^{-5}	7 (8·1)	16 (5·0)
Young animals, 17–18 g.	20	As above	10^{-5}	3 (7·3)	17 (5·2)
„ „ 17–18 g.	20	300 mg./kg. 48 and 150 mg./kg. 4 hr. before virus	10^{-3}	0	18 (5·4)

The Table shows the deaths in treated and untreated animals, with the mean periods of survival of fatal cases in parentheses. In all instances, control groups of mice of the usual weight (16–17 g.) treated with comparable doses of mepacrine showed the usual therapeutic response. Infection was by the intramuscular route, dosing oral.

In some experiments the mean period of survival was slightly increased in treated animals. The young *rabbit* rarely develops nervous illness (Hurst, 1950); if anything, its susceptibility is heightened by exhibition of mepacrine.

Because of the low incidence of encephalitis in the young *rabbit*, and in order to obtain the maximum amount of information with moderate expenditure in the *monkey* (*M. mulatta*), we decided to follow the course of the extraneural infection by daily titration of the blood of treated and untreated animals. In the mouse, treatment with mepacrine very markedly reduces the titre of virus circulating in the blood stream in the pre-encephalitic phases of the disease (Hurst, Melvin and

Peters, 1952). The present observations suggested that in the rabbit mepacrine may partially suppress the systemic growth of virus, without however diminishing the incidence of encephalitis and death. In two monkeys there was some temporary restriction of viral growth with a larger inoculum of virus. With a small inoculum, however, the titre of virus was higher in two treated monkeys, both of which died of encephalitis, whereas two untreated controls suffered an inapparent infection.

In short, with the exception of the young rat, we have been unable to reproduce in other animal species the striking therapeutic effect produced by mepacrine on equine encephalomyelitis in the mouse. From what we have written of the morphological changes following administration of mepacrine to these other species, it is clear that no obvious relation exists between the capacity to deposit basophilic granules in various situations and the presence or absence of a therapeutic response to the drug. This conclusion is valid when applied to the range of animal species considered in this report; we may anticipate a future paper by stating that it does not apply to the results obtained with a number of different acridines in a single species of animal—the mouse.

The lack of therapeutic significance of the basophilic particles in the mouse.

One further point remains to be mentioned and relates to the question initially posed at time of discovery of the basophilic particles. In an attempt to determine whether or not the particles contain a therapeutically active substance, we devised an experiment to dissociate as far as possible the action of mepacrine or one of its earlier metabolites from that of the particles. Details appear in Table II. They may be interpreted as indicating that a dose of mepacrine closely

TABLE II.—*Effect of Different Regimes of Treatment with Mepacrine on Equine Encephalomyelitis in Mice.*

Treatment.	Deaths.
(*a*) None	21 (5·9)
(*b*) Mepacrine 5 mg./20 g. on alternate days for six doses; 4 days interval before infection	13 (6·9)
(*c*) Mepacrine 5 mg./20 g. once 4 hr. before virus	1 (4·0)
(*d*) Mepacrine, as (*b*) and (*c*) combined	2 (7·0)

The Table shows the deaths in groups of 30 treated and untreated animals, with the mean periods of survival of fatal cases in parentheses, under different regimes of medication. Drug was given orally, virus intramuscularly.

related in time to infection with the virus is extremely potent, whether basophilic particles be present or not, but that the mouse heavily laden with basophilic particles but not given a recent dose of mepacrine fares indifferently in the therapeutic test. If we can assume, as seems likely from the observations of ourselves and others, that some mepacrine persists in the organs for days after the last of a series of doses, the results in the group of animals last mentioned suggest at most an inferior degree of therapeutic activity for the basophilic material, and more probably that the moderate activity observed under the conditions specified is due to persisting mepacrine.

SUMMARY.

The tissues and organs of mice given single or repeated large doses of mepacrine are coloured yellow for many days; this yellow colour is associated with the deposition in the reticulo-endothelial system, liver, kidney, muscle, skin, etc., of basophilic particles, the appearance and reactions of which are described in detail. When unstained the particles are yellow, and they fluoresce in ultraviolet light. Most of the nervous system does not share in the "persistent" macroscopic colouration, and basophilic particles are found only in the choroid plexuses and ventricular ependyma which are so coloured. The staining and biological properties of the particles suggest that they contain an acidic metabolite of mepacrine.

Various animal species differ greatly in their ability to become "persistently" coloured after mepacrine, and to deposit basophilic particles at the sites mentioned. Instead of basophilic particles they may show yellowish granules fluorescing in ultraviolet light. The picture in the rat, rabbit, guinea-pig, dog, monkey and chick are described.

The striking therapeutic activity of mepacrine against infections with eastern equine encephalomyelitis in mice is not reproduced in guinea-pigs, rabbits, chickens, monkeys or newly weaned rats; a response may be demonstrated in the adolescent rat. The therapeutic effect is not obviously related to the capacity or otherwise of the species to deposit basophilic particles after a dose of mepacrine. Evidence is presented that in the mouse the basophilic particles themselves have no great therapeutic activity.

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THE EFFECT OF *SALM. TYPHI* AND ITS ENDOTOXIN ON THE PHAGOCYTIC ACTIVITY OF THE RETICULO-ENDOTHELIAL SYSTEM IN MICE.

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CONSIDERABLE indirect evidence has been presented by Good and Thomas (1952, 1953) connecting the reticulo-endothelial system with the defence of the body against the toxins of Gram-negative bacteria. These authors have observed that rabbits whose R.E.S. has been blocked with either thorotrast, carbon or saccharated iron oxide are much more susceptible to damage by meningococcal toxin. A single injection of this toxin 6 hours after the administration of the blocking colloidal suspension results in the development of bilateral cortical necrosis of the kidney and death of the majority of the rabbits. The mechanism by which the normal activity of the cells of the R.E.S. protects against these injuries is not known. Moreover, experimental data are lacking concerning the changes in the activity of the R.E.S. brought about by one or more injections of Gram-negative bacteria or their endotoxin.

Endotoxins which are phospholipid-polysaccharide-protein complexes can be extracted from Gram-negative bacteria (van Heyningen, 1950). These complex toxins are structural components of the bacterial cell and are the dominant O somatic antigen of the smooth strains. All the toxic reactions caused by the bacteria themselves can be provoked by the isolated phospholipid-polysaccharide-protein complex. It is well known also that a previous non-lethal injection of one of the endotoxins protects the animals 24 hours later against several lethal doses of any other endotoxins (Cluff, 1954; Thomas 1954). The mechanism of this protection, which is not an immunity effect, is not clear, and it is a matter for speculation whether the R.E.S. plays a part in this protection. In view of these observations which have been also verified in our laboratory, it seemed of interest to investigate quantitatively the effect of a Gram-negative bacillus, *Salmonella typhi*, and of its endotoxin, on the functional activity of the R.E.S. measured with the methods which we have described.

In previous studies (Halpern, Benacerraf and Biozzi, 1953; Biozzi, Benacerraf and Halpern, 1953) a method was presented for investigating in laboratory animals the functional activity of the R.E.S. by measuring the rate of clearance from the blood of carbon particles of known size (250 Å) injected intravenously. Under these conditions, the R.E. cells of the liver and spleen were found to be responsible for more than 90 per cent of the phagocytic activity.

The clearance of all particulate matter from the blood stream follows an

exponential equation $C = C_o\ 10^{-KT}$. This is a general relationship valid for other substrates besides carbon (Benacerraf, Stiffel, Biozzi and Halpern, 1954). The constant K of the equation was called the phagocytic index,* and it is a measure of the functional activity of the R.E. cells of the animal for a given dose of carbon. It should be noted, however, that this index varied inversely with the dose of carbon injected. The same dose of particulate matter should be always administered in an assay of the functional activity of the R.E.S. In this case the index K is a true measure of the total phagocytic activity of the R.E.S. of the liver and spleen.

It was observed also that in rats and mice the phagocytic index for a given dose of carbon varied with the cube of the relative weight of the organs, liver and spleen, principally involved in phagocytic activity, according to the following equation

$$\alpha = \frac{W}{Wls}\ 3\sqrt{K}$$

where W is the weight of the individual animal and Wls the weight of the liver and spleen: α was called the corrected phagocytic index. It showed much smaller individual variations than the phagocytic index K itself.

As it is independent of the weight variations of the active phagocytic tissue, the index α is a measure of the phagocytic activity per unit weight of active R.E.S. tissue, while K measures the total phagocytic activity of the R.E.S. of the liver and spleen. The effect of the typhoid bacillus or of its endotoxin on the activity of the R.E.S. was investigated in mice by a study of the variations of the indices K and α for a standard dose of 16 mg. carbon per g. body weight.

EXPERIMENTAL.

The experiments were carried out on white male mice weighing about 20 g. A suspension of *Salm. typhi* killed by heat, prepared by the Pasteur Institute, was used throughout; 10^{10} organisms of this preparation had a dry weight of 1 mg. The endotoxin was prepared by the method of Boivin and Mesrobeaunu (1935, 1938) by Dr. Staub.

The test of the activity of the R.E.S. was carried out as described (Biozzi *et al.*, 1953). All animals were injected intravenously with 16 mg. carbon/100 g. body wt. of carbon suspension C11–1431a† prepared as described in our previous studies, and the clearance of carbon from the blood was measured. With this dose of carbon, the blood clearance in normal mice takes about 1 hr. Blood samples were obtained from the retro-orbital venous plexus with a calibrated (0·025 ml.) glass pipette previously washed with heparin. The plexus can be reached easily in mice in the inner angle of the eye. This technique permits the taking of numerous blood samples at regular intervals without injury to the animal. The blood was lysed in 2 ml. of 0·1 per cent Na_2CO_3 and the amount of carbon determined electrophotometrically in the red light. The phagocytic index K was calculated for each animal as described by Biozzi *et al.* (1953). The animals were sacrificed when all the carbon had been cleared from the blood. The spleen and the liver were weighed independently, the ratio W/Wls was calculated and the corrected phagocytic index α obtained from the equation cited in the introduction.

Eighty mice were used as controls; the activity of the R.E.S. was investigated in groups of these animals whenever treated mice were studied. All the results, means and standard deviations were calculated for this group of animals, they represent the control values found in Fig. 1 and 2 at 0 time.

* In previous papers we have used the term granulopectic index. We prefer now to use the term phagocytic to describe the same phenomenon.

† Gunther Wagner, Hanover.

RESULTS.

Effect of Intravenous Injections of Killed Typhoid Bacilli on the Activity of the R.E.S.

These experiments were carried out on 77 mice. All animals received a first injection of 5×10^9 bacilli into the dorsal vein of the penis. This dose of bacteria was not far from the lethal dose, which was found to be 10×10^9 bacilli. In the hours and days following this injection the activity of the R.E.S. was investigated in groups of animals. After 48 hr., the remaining animals received a second intravenous injection of 5×10^9 bacilli, and again the activity of the R.E.S. was investigated in groups of these mice in the hours that followed. On the 4th day the remaining animals received a last injection of 7×10^9 bacilli. On the 5th, 6th, and 9th day the remaining animals were studied. The mean phagocytic index K, the mean relative weight of the liver and spleen W/Wls and the mean corrected phagocytic index α were calculated for each group at each time. The results of these experiments are presented in Fig. 1. A histological study of the liver and spleen was carried out on several animals of each group.

The data presented in Fig. 1 show that in the hours that follow an intravenous injection of a large dose of killed typhoid bacilli the phagocytic activity of the R.E. cells of the liver and spleen is greatly depressed. Both the phagocytic index K and the corrected index α, which is a measure of activity per unit weight of active tissue, fall far below the limits of the normal standard deviation. The maximum effect is observed 10 hr. after the administration of the bacteria and it is still evident after 24 hr. The ratio W/Wls remains unchanged during this first phase of the experiment. All animals were in very good condition when studied and showed no evidence of shock.

When, after two days, the R.E.S. has recovered its normal phagocytic activity, a second dose of bacteria no longer causes a depression, but on the contrary, a marked stimulation of the phagocytic activity of the R.E.S. is observed ; 24 hr. after the second intravenous injection of 5×10^9 *Salm. typhi*, the index K is considerably increased ; its value differs from the control values by 5 times the normal standard deviation. The corrected index α is increased also, but relatively less than K because, under the influence of the bacterial products, the relative weights of the liver and spleen have increased. A third injection of bacteria on the 4th day does not increase this effect further. Following the last injection of bacteria, the phagocytic index decreases gardually but remains still slightly above normal on the 9th day. The corrected index α has by then recovered its normal value. The relative weights of the liver and spleen have remained greater than normal. The increased phagocytic activity of the R.E.S. after the second injection of bacteria can be analysed into : (1) an increase of activity per unit weight of liver and spleen, and (2) an increase in the size of these organs. After the 9th day the tissue is no longer hyperactive, but the relative hypertrophy of the liver and spleen remains, accounting for an index K somewhat higher than normal. Therefore repeated intravenous injections of *Salm. typhi* cause a considerable and sudden increase of the activity of the R.E.S. per unit weight of tissue which is of short duration and also an increase in the size of the liver and spleen which is more lasting and which may be due to an increase in the number of cells.

It should be stressed that the effect of a single and of two intravenous injections of typhoid bacilli is very different. The first injection causes a very severe

depression of phagocytic activity. Histological study of the liver shows that the Kupffer cells have not taken up the carbon regularly. There are areas where the phagocytes have not absorbed carbon at all (Fig. 3 and 4). Some cells seem to have been injured by the germs phagocyted. It is very interesting that a second dose of bacteria in an animal having received a previous equal dose 48 hr. before, no longer causes injury to the cells, but on the contrary, stimulates them greatly. The protection seems to come much too early to be an immunity effect; it is associated with an increased resistance of the animals to the endotoxin of the bacteria. The mice tolerate at that time up to three times the normal lethal dose of endotoxin.

Effect of the Intravenous Injection of the Boivin Endotoxin on the Activity of the R.E.S.

The experiments with the Boivin endotoxin were carried out on 10 mice. The lethal dose of the preparation was 300 γ. A single intravenous injection of 150 γ of the preparation was given to 5 animals, and the activity of the R.E.S. was investigated 20 hr. later. Another 5 animals received a first injection of 75 γ followed 24 hr. later by 300 γ. These two injections were very well tolerated. The activity of the R.E.S. was investigated in this last group 24 hr. after the last injection of the preparation. The indices K and α were calculated for each group. The results are presented in Table I.

TABLE I.—*Effect of* Salm. typhi *Endotoxin on the Phagocytic Activity of the R.E.S. Measured in Mice by the Indices* K *and* α *for the Dose of* 16 *mg. Carbon per* 100 *g.*

Control mice.		Endotoxin 150 γ I.V. carbon 20 hr. after single dose.		Endotoxin first dose 75 γ I.V.; second dose 300 γ I.V. 24 hr. later. Carbon 24 hr. after second dose.	
K.	α.	K.	α.	K.	α.
0·022±0·011	4·4±0·5	0·007	3·4	0·044	5·8
		0·006	3·6	0·043	5·8
		0·008	3·4	0·035	6·4
		0·007	3·5	0·028	5·1
		0·013	3·8	0·049	5·9
		Mean 0·008	Mean 3·5	Mean 0·04	Mean 5·8

Twenty hr. following the intravenous injection of 150 γ of endotoxin the activity of the R.E.S. is greatly decreased, just as after the injection of 5×10^9 killed bacteria (Fig. 1). The index K falls from 0·022 to 0·008.

Just as in experiments with bacillary suspensions, the reaction was different after repeated injections of the endotoxin. A previous injection of 75 γ 24 hr. before enables the animal to tolerate a much higher dose of the endotoxin. In this case repeated injections of the endotoxin stimulate the phagocytic activity of the R.E.S. Both K and α are found elevated far beyond the limits of the standard deviation of the controls. Although the intensity of the effects observed following the injection of the endotoxin are below those of the killed bacilli, the pattern of the action is identical. The differences in intensity may be due to the way the endotoxin is split from the bacteria in the animal, or to differences between

the way in which the endotoxin or the whole bacteria are taken up by the cells of the R.E.S.

It follows from these experiments, nevertheless, that the Boivin endotoxin of *Salm. typhi* is quite readily taken up by the cells of the R.E.S.; it is both able to cause injury to these cells and to stimulate their activity just as whole bacilli do. Therefore it would seem that the effect of the latter on the R.E.S. shown in Fig. 1 can be attributed to the endotoxin complex which is responsible for both the toxicity and the O somatic antigen.

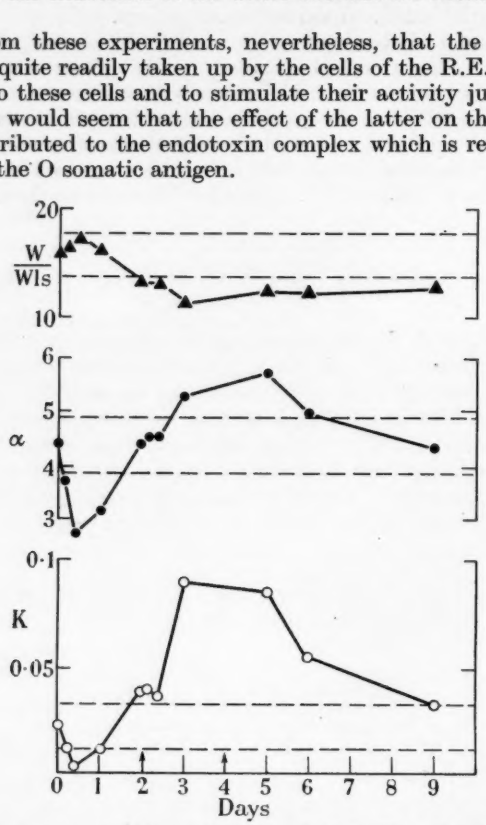


FIG. 1.—Effect of intravenous injections of killed typhoid bacilli on the phagocytic activity of the R.E.S. for the dose of 16 mg. carbon/100 g. body wt. and on the relative weights of the liver and spleen in mice. Arrows indicate time of injections of bacilli (5, 5, and 7 $\times 10^9$). Broken lines indicate the limits of the normal standard deviations.

As the endotoxins of Gram-negative bacteria are phospholipid-polysaccharide-protein-complexes, it seemed of interest to investigate whether the polysaccharide could be responsible for any part of the effect of whole complex on the R.E.S. Experiments performed with polysaccharide extracted by acid hydrolysis on 12 mice injected with doses of polysaccharide as high as 300 γ three times every two days indicate that this fraction of endotoxin is not responsible for the action of endotoxin on R.E.S.

Effect of Subcutaneous Injections of Killed Typhoid Bacilli on the Activity of the R.E.S.

These experiments were carried out on 35 mice. All animals received an injection of 5×10^9 of killed *Salm. typhi* subcutaneously. All animals withstood

this injection very well. In the hours and days following this injection the activity of the R.E.S. was investigated in groups of animals. On the second day the remaining animals received 5×10^9 and on the 4th day 7×10^9 bacilli subcutaneously, *i.e.*, same dosage schedule as was used in the experiments employing intravenous injection. The phagocytic activity of the R.E.S. towards carbon particles was investigated again 24 hr., 2 days, 3 days and 5 days after the last injection. The index K, the value W/Wls and the index α were calculated for each group at each time. The results of these experiments are presented in Fig. 2. A histological study of the liver and spleen was carried out in selected animals of each group.

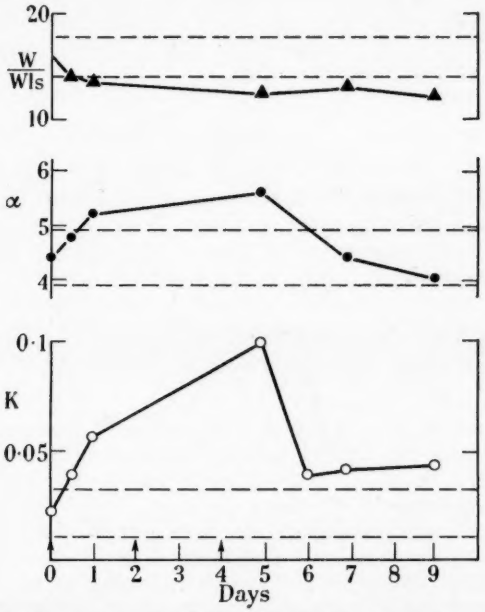


FIG. 2.—Effect of subcutaneous injections of killed typhoid bacilli on the phagocytic activity of the R.E.S. for the dose of 16 mg. carbon/100 g. body wt., and on the relative weights of the liver and spleen in mice. Arrows indicate time of injections of bacilli (5, 5, and 7 $\times 10^9$). Broken lines indicate the limits of the normal standard deviations.

It will be seen that killed typhoid bacilli administered subcutaneously cause a very marked increase in the phagocytic activity of the R.E.S. This stimulation is already evident after the first injection. The noxious action on the R.E.S. observed after a first intravenous injection of the same dose (Fig. 1) does not occur when the bacilli are injected subcutaneously; the reasons for these differences will be discussed later. Apart from these early differences the same doses of killed bacilli ultimately produce the same hyperactivity of the R.E.S. when injected by either route. The index K calculated from the values of 9 mice reached 0·1, 24 hr. after the last injection while that of the 80 controls was 0·22.

The corrected index α was also increased, but less than K, because subcutaneous

injections also cause a significant increase in relative weight of the liver and spleen, comparable with that observed after intravenous injection. The effect of multiple injections of killed *Salm. typhi* intravenously or subcutaneously is first a transitory increase of the activity of the R.E. cells of the liver and spleen per unit weight of tissue and second a more durable increase in the size of these organs and thus in the total activity of the R.E.S.

Injections by both routes caused a transitory loss of weight of about 10 per cent, but within 4–5 days after the last injection the animals had recovered from this and had even gained weight. The final and constant increase in relative weight of the liver and spleen was thus absolute and not the result of wasting. Histological study of the liver has also shown that the products of bacilli injected subcutaneously bring about the same type of picture as intravenous injection. At the time of the highest stimulation of the R.E.S. there appears to be an increase in the number of phagocytes in the liver.

The Effect of Cortisone on the Stimulating Action of Killed Typhoid Bacilli on the R.E.S.

These findings show that the R.E.S. reacts to repeated aggressions with the bacterial products of *Salm. typhi* by a marked increase in activity, which may be explained in part by an increase in the number of phagocytic cells. On the other hand, it is now well established that cortisone reduces considerably both the intensity of inflammatory reactions and resistance to infection. It has been reported (Benacerraf, Halpern, Biozzi and Benos, 1954) that in rats cortisone in well tolerated doses did not affect the phagocytic activity of the R.E.S. for carbon particles, but that it prevented the R.E.S. from recovering its normal activity after a blocking dose of carbon.

It seemed of interest to investigate in the course of these experiments whether cortisone inhibits the response of the R.E.S. to multiple injections of killed typhoid bacilli, thus possibly throwing some light on the mechanism by which cortisone lowers resistance to infection.

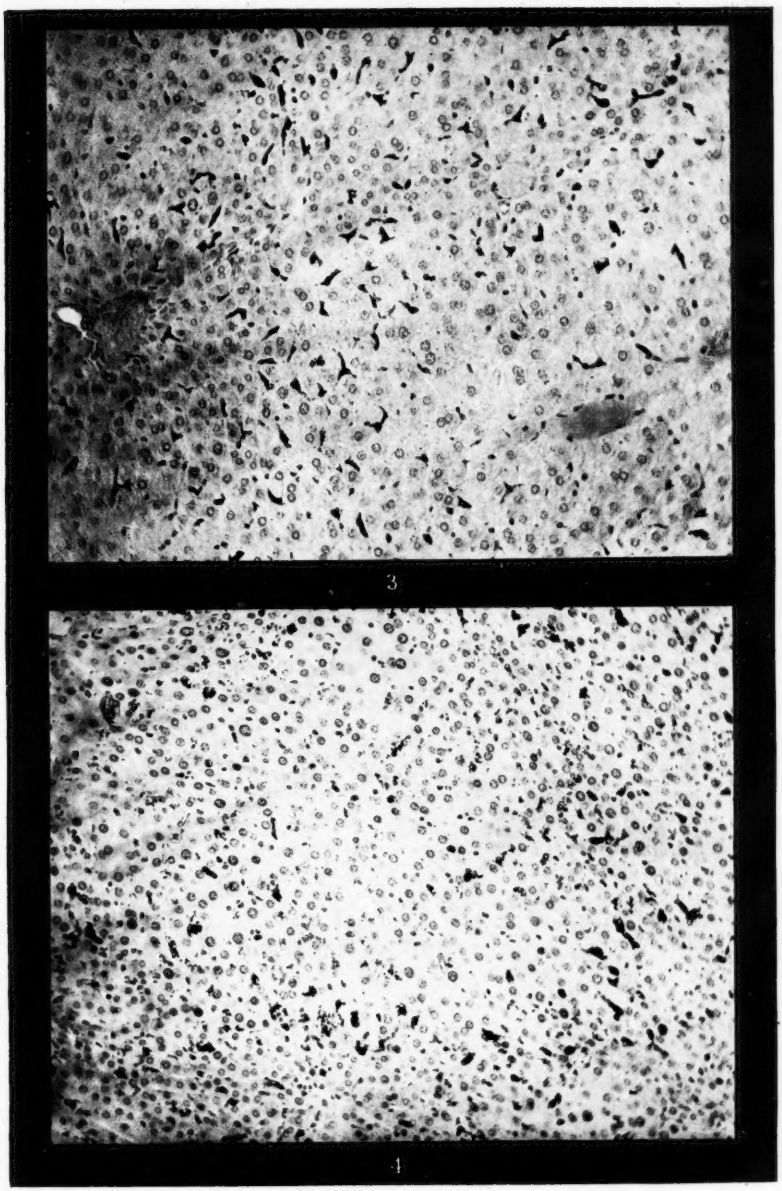
The experiments were carried out in three groups of mice. The first group (20 mice) received 3 injections of killed typhoid bacilli intravenously every two days, using the same dosage as in the earlier experiments. The second group (13 animals) received the same course of treatment with killed bacilli intravenously and also cortisone 1 mg. per day starting the day before the first injection of *Salm. typhi* (total 6 days). The activity of the R.E.S. was investigated in these two groups 24 hr. after the third and last injection of bacilli, which is the time when the activity had been found to be highest.

A last group of 8 mice received cortisone alone, 1 mg. per day for 6 days, in order to investigate also the effect of this dose of cortisone alone on the phagocytic activity of the R.E.S. The results of these experiments are presented in Table II

EXPLANATION OF PLATES.

FIG. 3.—Liver of a control mouse given standard dose of carbon intravenously and killed 2 hours later. Carbon is evenly distributed in Kupffer cells throughout. × 300.

FIG. 4.—Liver of a mouse injected with 5×10^9 killed *Salm. typhi* intravenously. This animal received the standard dose of carbon 10 hr. later, and was killed 2 hr. after carbon injection. Carbon is not evenly distributed in Kupffer cells. Many phagocytes have not taken up the carbon particles. × 300.



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as the mean phagocytic index K for each group with its standard deviations. The corrected index α was not calculated because cortisone causes independent changes in the weight of the liver due to accumulation of glycogen and fats, and in the weight of the spleen due to destruction of lymphocytes.

It should be mentioned first that cortisone is not as well tolerated by mice as it is by rats. Rats tolerated 5 mg. daily for periods of two weeks without mortality and with only a loss of weight of about 15 per cent, while in mice 1 mg. per day, which is an equivalent amount, causes an increasing percentage of mortality if administered for more than a week.

The results (Table II) show that treatment with cortisone (1 mg. per day for 6 days) largely inhibits the stimulating effect of repeated doses of typhoid bacilli on phagocytic activity. The figures are clearly outside the standard deviations for each group. The hormone, however, is not able to nullify completely the

TABLE II.—*Effect of Cortisone and the Response to killed* Salm. typhi.

	Controls.	Cortisone alone 1 mg./day × 6.	*Salm. typhi* 3 injections.	Cortisone 1 mg./day × 6. *Salm. typhi* 3 injections.
Number of mice	80	8	20	13
Phagocytic index K	$0{\cdot}022 \pm 0{\cdot}011$	$0{\cdot}015 \pm 0{\cdot}010$	$0{\cdot}085 \pm 0{\cdot}035$	$0{\cdot}038 \pm 0{\cdot}010$

stimulating effect of the bacterial products. On the other hand, the effect of cortisone on the normal phagocytic activity of the R.E.S. towards carbon is not striking. Although the average for the 8 animals treated with the hormone is slightly below the control values, the mean values obtained are well within the standard deviation of the control animals. Nevertheless, on the basis of experiments, prolonged treatment with cortisone may have in mice a slight inhibitory effect on the normal phagocytic activity of the R.E.S., while the same treatment has no effect in rats (Benacerraf, Halpern *et al.*, 1954).

DISCUSSION.

When either killed typhoid bacilli or their endotoxin are injected intravenously in mice the same effects are observed on the R.E.S., so that the action of the bacilli can be attributed at least partly to the endotoxin. After a first intravenous injection of either, the R.E. cells of the liver and spleen appear to have suffered ; their phagocytic activity is considerably depressed. However this first injection conditions the animal so that a subsequent injection no longer causes the same effect, but is followed by a considerable increase in activity of the R.E.S. It may be that a first contact makes the R.E. cells better able to destroy the toxin subsequently.

This difference in behaviour of the R.E.S. after a second injection of toxin coincides with a greater tolerance of the animal for the toxin. There is thus a time relationship between the more favourable reactions of the R.E.S. to the intravenously administered toxin and the greater resistance of the animal. These results added to the experiments of Good and Thomas, discussed above, suggest that the R.E.S. plays a definite rôle in the defence of the animal against endotoxins

of Gram-negative bacteria. Another strong point in favour of this interpretation is that the toxin seems to be taken up by the cells of the R.E.S., since it is able to affect them in such a definite way.

When the same dose of bacilli is administered subcutaneously this initial depression is not observed ; on the contrary, there is greater activity even after the first injection. It is possible that by the subcutaneous route not enough toxin reaches the R.E. cells of the liver and spleen to harm them, but enough to stimulate them. The fact that subcutaneous injection stimulates the phagocytic activity of the R.E.S. just as well as intravenous is very interesting, because it brings more proof that the split products of the bacilli are responsible for their stimulating effect on the R.E.S.

The increased phagocytic activity of the R.E.S. excited by bacterial products is the result of two factors : (1) an increased activity of the R.E. tissue per unit weight which is transitory, and (2) an increase in size of the liver and spleen which is more lasting. To what factors should this increase in weight be attributed ? Since it is directly related to a stable increase of function, one may suggest that there has been an increase in the number of cells under the stimulating effect of the bacterial products. A similar increase in liver and spleen weight associated with an increase in R.E.S. function was also observed in mice in the course of experimental tuberculosis (Biozzi, Benacerraf, Grumbach, Halpern, Levaditi and Rist, 1954).

It seems that prolonged treatment with cortisone in the dosage used may have in mice a slight depressive action on the normal phagocytic activity of the R.E.S. for carbon particles, while the same dosage has no effect in rats (Benacerraf, Halpern *et al.*, 1954). With much higher doses in rats however, Heller (1953) observed that cortisone lowers the normal phagocytic activity of the R.E.S. towards radioactive chromium phosphate. It should be stressed that mice do not tolerate treatment with high doses of cortisone as well as rats. The inhibitory effect of cortisone on the stimulation of the R.E.S. by killed typhoid bacilli is much easier to demonstrate. It illustrates again the mechanism by which this hormone renders animals less resistant to infections. Cortisone probably inhibits the cellular response of the R.E.S. caused normally by bacterial attacks, and in this way it impairs the defences of the animal against invading bacteria.

SUMMARY.

The effects of injections of killed *Salm. typhi* or of its endotoxin on the phagocytic activity of the R.E.S. were investigated in mice by determinations of the rate of disposal of intravenously injected carbon.

A single large dose of bacilli or endotoxin administered intravenously causes a transient depression of the phagocytic activity of the R.E.S.

Multiple intravenous or subcutaneous injections bring about a very marked increase in phagocytic activity, and also a significant increase in the relative weights of the liver and spleen. At the same time the resistance of the animals to the toxic products of the bacilli is substantially increased. The significance of these facts in relation to the rôle of the R.E.S. in defence against bacterial endotoxins is discussed.

Treatment with cortisone greatly reduces the stimulating effect of the killed typhoid bacilli on the phagocytic activity of the R.E.S.

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OBSERVATIONS ON THE EFFECT OF ISONIAZID AND STREPTOMYCIN ON TUBERCLES IN A TRANSPARENT CHAMBER IN THE RABBIT'S EAR.

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THE effects of the administration of isoniazid and streptomycin given singly and in combination have been observed on tubercles growing in transparent chambers in the rabbit's ear. A description of the development of tubercles in such chambers was given by Dodson, Sanders and Florey (1954). Some experiments, for comparison, were done on rabbits with extensive miliary tuberculosis.

A. EXPERIMENTS USING THE EAR CHAMBER.

Production of tubercles in the ear.

The apparatus and procedures used were those described by Sanders, Dodson and Florey (1954). Tubercles were induced in the thin transparent tissue of the rabbit ear chamber by introducing the " Branch " strain of bovine *Mycobacterium tuberculosis*.

Administration and dosage of drugs.—Three groups of animals were treated: one with isoniazid alone, another with streptomycin alone, and the third with the two drugs together. The drugs were dissolved in 1·0–1·5 ml. of sterile distilled water and were given subcutaneously morning and evening into the back or thigh. Isoniazid in doses of 25–30 mg./kg. body wt. did not produce toxic signs. Larger doses did not give a better therapeutic response. The one rabbit to which 40 mg./kg./day was given became restless and excitable, lost weight and refused food and water. Streptomycin was given in doses of from 50–100 mg./kg. daily without evidence of toxicity. The same doses were given when the two drugs were used together. They were administered as above, and were injected separately.

Post-mortem investigations.—When the area being observed had become caseous the rabbits were killed by the rapid intravenous injection of nembutal. The skin around the chamber was shaved and painted with iodine and alcohol, and the lid of the chamber was prised off. The caseous material on the table or sticking to the centre of the coverslip was collected as completely as possible on a wire loop. This was covered with fine polythene tubing to prevent the perspex surface of the chamber from being scratched by the wire. Tissue for histological section was taken from the part of the ear adjacent to the edge of the table.

The caseous material from the first three rabbits, which were treated with isoniazid, was cultured in fluid Tween-albumin medium. The tubercle bacilli which grew were tested for resistance to this drug by subculture into the same medium containing doubling dilutions of isoniazid. The caseous material from all other animals was dispersed in 0·1 per cent bovine albumin by gentle grinding in a Griffith's tube, and dropped in various dilutions on the surface of oleic acid-albumin agar plates. To estimate whether drug resistance had developed, some of the plates contained various concentrations of isoniazid or streptomycin or both. After the plates had been incubated the number of colonies which formed was counted as in Fenner's (1951) technique. Penicillin in a concentration of 2–5 units/ml. was added to all plates. It

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was repeatedly found, as others have shown, that the addition of penicillin in these concentrations did not affect the growth of the tubercle bacilli.

In most cases guinea-pigs and/or rabbits were inoculated with diluted caseous material dispersed in bovine albumin to ascertain whether viable tubercle bacilli were present.

RESULTS.

Isoniazid.

Five animals were treated with isoniazid alone. Tubercles were treated either before or after caseation appeared. The lesions in the ear chambers continued to enlarge for 3 or 4 days after treatment had begun, but in each case the spread stopped on the 4th day of treatment. After this, the size of the lesion began to decrease.

The degree and rapidity of healing depended on the presence or absence of caseous material in the tubercle. A lesion which had not yet caseated disappeared rapidly under treatment, but even a small amount of caseous material greatly delayed resolution. One caseous tubercle, 0·7 mm. in diameter, actually did resolve completely, but the other caseous tubercles studied, which were 2 mm. or more in diameter, did not resolve completely. Without exception, all the caseous lesions began to expand again between the 26th and 34th days of observation, despite continuing treatment.

Viable tubercle bacilli persisted in all animals, even those in which caseation had not occurred and in which all microscopical signs of the lesion had disappeared. Although treatment was continued for as long as two months after the complete disappearance of the lesions, fresh tubercles appeared a few weeks after treatment was stopped, either at the original site or elsewhere in the chamber. One such recurrence was treated with isoniazid and began to heal again ; 29 days later, however, isoniazid ceased to be effective and the tubercle spread rapidly across the chamber.

Tubercle bacilli were cultured from all these chambers, but it was not possible in the liquid Tween-albumin medium which was used for material from the first 3 chambers to demonstrate resistant organisms. Organisms resistant to 2 μg. of isoniazid per ml. of medium were isolated from a fourth chamber, when the caseous material was inoculated on oleic acid-albumin-agar. Material from the fifth chamber became contaminated during collection and could not be tested for resistance.

The biological state of the organisms from a lesion which had begun to spread despite continuing isoniazid therapy was investigated as follows. Part of the caseous material from such a lesion was homogenised in bovine albumin and inoculated into a normal chamber. Although a tubercle developed in this chamber its behaviour was atypical. A lesion appeared fairly rapidly, but was slow to progress and remained a loose collection of macrophages. Caseation did not occur until a focal tuberculin reaction was induced by the intravenous injection of P.P.D. (purified protein derivative of tuberculin). Even after caseation the lesion extended slowly and in three months had not completely spread across the table of the chamber. A course of isoniazid failed to cause regression. Caseous material from this chamber was injected into a rabbit, and a few tuberculous foci were later found in its liver and spleen. However, on oleic acid-albumin-agar the material produced no growth.

Organisms made resistant to 100 μg. of isoniazid per ml. by serial culture in Tween-albumin medium containing ascending concentrations of isoniazid were introduced into a normal chamber. Again an indolent and atypical lesion resulted. The rate of spread of this lesion was about four times slower than the usual tubercle in an ear chamber. The lesion did not respond to isoniazid treatment. Although tubercle bacilli were seen after death in direct smears of material from the tubercle, these bacilli did not grow on oleic acid-albumin-agar and failed, on inoculation, to infect either guinea-pigs or rabbits. That this lesion in the ear chamber was in fact a tubercle was shown by the development of a positive cutaneous tuberculin reaction in the animal.

The above experiments indicate alterations in the virulence of isoniazid-resistant organisms and in their ability to grow on artificial media. These conclusions are in agreement with those of Middlebrook and Cohn (1953), Barnett, Bushby and Mitchison (1953), Fisher (1952) and Karlson and Ikemi (1952).

Streptomycin.

Three animals were treated with streptomycin alone. Two of these, one with a small non-caseous lesion and the other with a large caseous tubercle, were given 50 mg. of streptomycin per kg. body wt. daily for 9 and 15 days respectively. In this dosage the drug had no effect on the microscopical appearance of the lesions or on their rate of spread. The lesions spread across the area of observation in 20 and 21 days respectively. A possible explanation for this lack of effect is offered by the work of Steenken and Pratt (1949). These authors showed that the early (up to 27 days) dissemination of tubercle bacilli in guinea-pigs was not affected by streptomycin therapy and that there was no sign of healing until the animals reacted to tuberculin and had presumably acquired resistance to the organisms.

Effect of immunisation.

Steenken and Wolinsky (1947) showed that vaccination with avirulent tubercle bacilli (H37Ra) favourably influenced the results of streptomycin treatment. Jensen (1949) came to a similar conclusion. Accordingly, a rabbit was immunised with the Copenhagen strain of B.C.G. A 6-days-old culture of this organism in liquid Tween-albumin was diluted 1/100 in 0·1 per cent bovine albumin, and 0·3 ml. of this dilution was injected intravenously. A second injection was given five weeks later, when the animal was strongly tuberculin-positive. The chamber was inoculated on the next day.

When a tubercle appeared, streptomycin treatment (50 mg./kg. body wt. daily) was begun. The lesion progressed in the usual manner for about a week, but by the 7th day of treatment its appearance had come to resemble that of a lesion responding to isoniazid therapy. (See later under " Observations on the mode of healing of tuberculous lesions".) Nevertheless, the tubercle continued to expand, and by the 55th day of treatment about half the area of observation was necrotic. The dose of streptomycin was then raised to 100 mg./kg. daily. Six days later the lesion began to heal rapidly. For a week the speed and extent of healing was most striking, and greater than had been observed in any other chamber. However, during the next few days thrombosis occurred in most of the vessels in the chamber, leading to the necrosis of the entire area of observation (Fig. 1–3). Despite this event, the cause of which is unknown, it appears likely that strepto-

mycin, in the presence of acquired resistance, had retarded the spread of the lesion and had enabled healing to occur for a time.

Isoniazid and Streptomycin.

Four rabbits were treated with the two drugs together. In two animals the chambers contained small tubercles when treatment was begun. These appeared to heal completely, but fresh lesions appeared after the treatment was stopped, even though it had been continued for two months after apparent healing. In one animal treatment was recommenced, but the relapsed lesion continued to expand very slowly. It had not spread completely across the chamber in 210 days after its appearance.

In two animals with ear chambers containing large caseous tubercles some healing occurred, but, as with the two drugs separately, healing was never complete. The rate of progress of the lesions was, however, retarded (Fig. 4–6).

In one animal, only a minute tubercle remained after twelve months' treatment. A month after treatment was stopped this chamber began to separate from the ear. As separation became more marked one of the main vessels at the edge of the chamber thrombosed, producing infarction of the whole table area. Such an event is often seen in old chambers and in this case appeared to be unrelated to the tuberculous infection.

Bacteriological observations.

Although tubercle bacilli were demonstrated in material from all these chambers by one method or another, their growth on oleic acid-albumin-agar was capricious. Cultures on this medium were obtained from two chambers. In both cases the organisms were sensitive to isoniazid and streptomycin.

Conclusion.

Isoniazid and streptomycin used in combination held tuberculous infections in check for longer periods, and allowed greater healing to occur than when either drug was given alone. Lesions treated with the two drugs in combination also showed less tendency to relapse.

B. EXPERIMENTS USING ANIMALS WITH MILIARY TUBERCULOSIS.

As it is possible that tubercles growing in the subcutaneous tissue of the ear might behave differently from tubercles in the viscera some experiments were done using animals which had been infected intravenously.

Methods.

Twelve rabbits weighing about 2 kg. were infected with the Branch strain of *Myco. tuberculosis*. A 6-days-old culture in Tween-albumin medium was diluted 1/1000 in the same medium, and 0·5 ml. of this dilution was injected into the marginal ear vein. This procedure produces extensive miliary tuberculosis ; the rabbit usually dies between 25 and 35 days after infection. To make sure that lesions were present radiographs of the lungs were taken twice weekly with a portable X-ray machine.

Two animals died of intercurrent infection (pneumonia and coccidiosis) before tuberculous lesions could be found. Numerous miliary lesions were seen in the radiographs of the other animals three weeks after infection. The number and size of the tubercles appeared much the same in all animals.

One animal was killed at this time to provide control histological material. The remaining 9 rabbits were divided into three groups. One group was given 30 mg. isoniazid/kg. daily, the second 50 mg. streptomycin/kg. daily, and the third 30 mg. isoniazid and 50 mg. streptomycin/kg. daily.

RESULTS.

The progress of the miliary tuberculosis was rapidly halted by isoniazid, and by isoniazid and streptomycin in combination. Streptomycin given alone prolonged the life of one animal and its lesions showed signs of partial arrest.

In all treated animals (except two which died with confluent miliary lesions before therapy could have any effect), the number of tubercle bacilli seen in histological preparations of the lesions was greatly reduced compared with the untreated control, and organisms were to be found only on the caseous centres of the tubercles.

EXPLANATION OF PLATES.

PLATE I.

Fig. 1, 2 and 3: Rabbit vaccinated with B.C.G. and treated with streptomycin.

FIG. 1.—Fifty-first day of treatment. This is the maximum size reached by the lesion. The dose of streptomycin was then doubled.

FIG. 2.—Fifteen days later. Some resolution has occurred and new blood vessels can be seen growing into the caseous material.

FIG. 3.—Four days later. Commencement of final relapse in spite of treatment. Vessels in stasis.

Fig. 4, 5 and 6: Combined treatment with isoniazid and streptomycin.

FIG. 4.—Twenty-first day of treatment. Blood vessels are growing in from the periphery at the right-hand side of the picture and necrotic tissue is beginning to be absorbed.

FIG. 5.—Hundredth day of treatment. A considerable amount of healing has taken place.

FIG. 6.—Two hundred and forty-first day of treatment. Further absorption of necrotic material. Fibrous tissue can be observed in the upper part of the picture.

(Magnification × 14.)

PLATE II.

Fig. 7, 8, 9 and 10: Treated and untreated miliary tuberculosis of the lung. Haematoxylin and eosin stain.

FIG. 7.—Lung of untreated rabbit three weeks after infection. The miliary tubercles have diffuse and ill-defined edges, and some are becoming confluent. × 40.

FIG. 8.—Lung of rabbit treated for three weeks with isoniazid. The tubercles show well-defined edges and calcification is present in the caseous centres of many lesions. × 40.

FIG. 9.—Lung of rabbit treated for 6 months with isoniazid and streptomycin. The tubercles have fibrous capsules. The calcified centres of many tubercles were torn out during section cutting, leaving empty spaces. × 40.

FIG. 10.—Higher magnification from same lung as Fig. 9, showing fibrosis and calcification. × 160.

PLATE III.

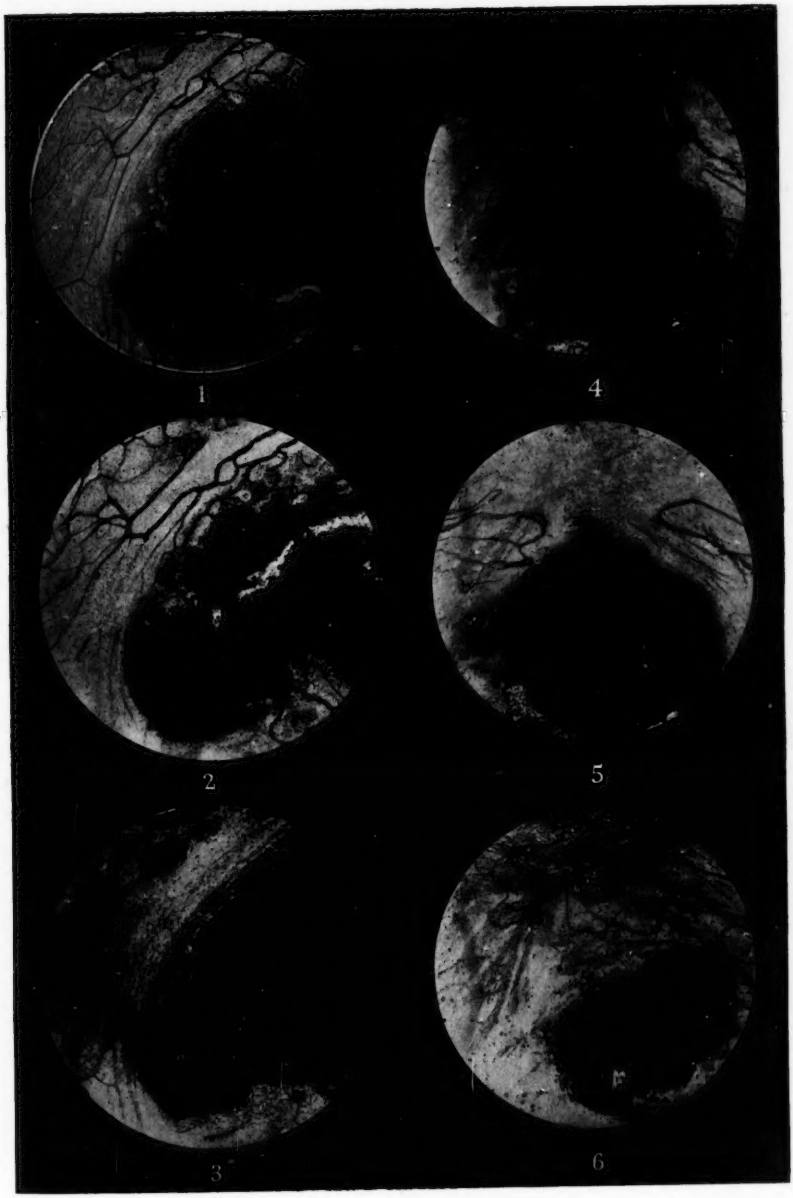
FIG. 11.—The edge of a resolving tubercle. The epithelioid cells are dark (stained vitally with Evans blue). They are no longer tightly packed as in earlier lesions but are well separated. × 170.

Fig. 12, 13 and 14: Vascular changes round a slowly healing tubercle under treatment with isoniazid. (× 14.5.)

FIG. 12.—A lesion that had been present for 21 days after 4 days of treatment; its spread has just ceased.

FIG. 13.—Tenth day of treatment. The encircling vessel is now tortuous and U-shaped segments are growing towards the tubercle.

FIG. 14.—Thirty-first day of treatment. The tubercle has begun to spread again and some of the vessels are thrombosed.



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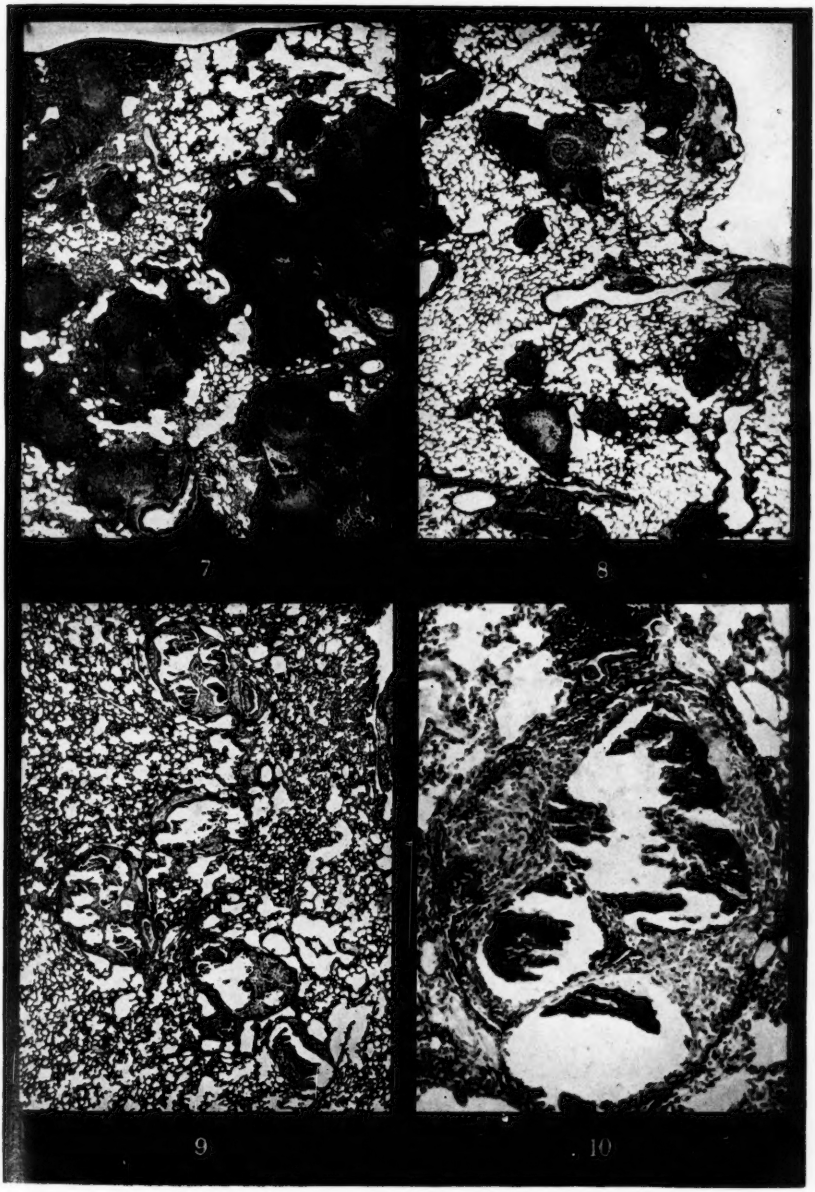

Dodson, Sanders and Florey.

Dodson, Sanders and Florey.

Healing was complete in, and organisms eradicated from, the liver, spleen and kidneys of one animal which received isoniazid and streptomycin for 26 weeks. The pulmonary lesions, however failed to heal and tubercle bacilli were recovered from this organ (Fig. 7–10).

There were clear differences in the response to treatment of lesions in the different organs. A striking feature in all animals was the early disappearance of caseous material from, and rapid healing of, hepatic lesions. The work of Soper (1917–18) and Rich and McCordock (1929), who described spontaneous resolution of tubercles in the livers of rabbits, makes it difficult to assess the importance of drugs in the rapid healing of the hepatic lesions seen in the present experiments.

It is clear that no close correlation can be established between the results of treatment of disseminated tuberculosis and of tubercles in the ear chamber. Perhaps the lesions in the ear chamber can be more closely correlated with the pulmonary lesions than with those in other organs.

This series of animals is too small to assess the relative merits of the treatments used.

OBSERVATIONS ON THE MODE OF HEALING OF TUBERCULOUS LESIONS.

The following observations on the healing of tuberculous lesions are recorded to supplement previous descriptions of the development and progress of tubercles in the rabbit ear chamber (Ebert, Ahern and Bloch, 1948 ; Ebert and Barclay, 1950 ; Sanders, Florey and Wells, 1951 ; Dodson *et al.*, 1954).

Initial reaction of healing.—The first sign that treatment was becoming effective was the failure of the tubercle to make its usual daily increase in size and cellularity. At the same time the blood flow in the vessels which had survived was restored to normal. Local oedema rapidly disappeared, and fewer leucocytes stuck to the vessel walls. It was no longer possible, once treatment had become effective, to produce intense staining of the epithelioid cells around the lesion with a single intravenous injection of vital dye. The most likely explanation of this finding is that the capillaries had recovered their normal permeability, and did not allow the dye to escape freely.

Almost immediately after the lesion had ceased to spread, capillary buds began to grow from the inner side of the adjacent vessels towards the lesion ; and the peripheral accumulation of cells, mostly polymorphs and macrophages lying outside the epithelioid cell barrier, began to disappear within two or three days.

The appearance of a healing tubercle at this stage, with capillary buds growing into it from the adjacent vessels, which carried a brisk flow close to the sharply demarcated edge, made it easily distinguishable from a spreading lesion which had ill-defined edges and a poor circulation in the adjacent vessels.

The same reparative processes were found in sections of organs from animals with miliary tuberculosis. They were particularly clear in the lungs, where the exudate in the surrounding alveoli was rapidly and completely absorbed. The lesions became circumscribed and were separated by areas of emphysematous alveoli which no longer contained exudate. New capillaries could be seen between the epithelioid cells at the margin of the tubercle.

These changes were seen whenever healing occurred, but the subsequent events varied with the effectiveness of chemotherapy and the presence or absence of caseous matter.

The resolution of small tubercles.—A number of small non-caseous tubercles

were seen to resolve completely during treatment. Cells disappeared first from the edge of the lesion. As their number progressively diminished, capillaries grew out between them. The reconstruction of the capillary bed was not complete until most of the cells had disappeared. Occasionally, in a very small tubercle, a vessel which had been in stasis began to carry blood through the lesion again.

Although several small caseous tubercles were seen to resolve, the caseous material was absorbed slowly. Macrophages phagocytosed the necrotic tissue, and the lesion then consisted of a group of large débris-laden phagocytes (macrophages or epithelioid cells). Capillaries finally grew across the area, but most of them disappeared later, leaving a mature vascular pattern.

The healing of large caseous tubercles.—The healing of these (with an exception in one streptomycin-treated animal) was slow and never complete, but the same general phenomena were seen. The layer of epithelioid cells decreased in width, and the cells separated from each other (Fig. 11).

Reactions of epithelioid cells.—If vital dye was injected before treatment began, the epithelioid cells stained diffusely. When treatment became effective some alteration took place in the cells so that the dye, during the course of a few days, was gradually segregated into granules (or vacuoles), and the cells stained in a punctate manner like histiocytes. Indeed, the process was precisely the same as that seen when histiocytes were stained vitally in a normal chamber (Ebert and Florey, 1939).

As healing progressed fewer and fewer epithelioid cells at the edge of the caseous material could be stained with vital dyes. Observation of individual epithelioid cells over some days gave no indication that they migrated any distance, although, like histiocytes, they changed shape and moved a few microns apparently at random. It seemed unlikely therefore that they disappeared by migration to the blood vessels. It was thought that they probably died *in situ* and were taken up in fragments by other phagocytes.

The manner of capillary ingrowth.—In some tubercles the vessels at some distance from the lesion went into stasis and an ischaemic, but not caseous, zone lay between the nearest vessels and the edge of the tubercle proper. When healing began in these cases, capillary sprouts were seen to grow into the lumina of the obstructed vessels and to form loops. As these loops elongated, the blood clot ahead of them resolved, and they grew along the lumen of the vessel. More commonly the circulation was still present at the edge of the lesion, and no such line of least resistance was offered to vascular growth. Capillary buds then formed on the vessel nearest to the lesion and grew between the epithelioid cells sometimes directly into the caseous matter. The buds were blind endothelial tubes, which usually contained a few leucocytes and rouleaux of red cells. Each bud lengthened and eventually anastomosed with an adjacent one to form a capillary loop, which in turn gave rise to further sprouts (Fig. 12–14).

Only on a few occasions, when the lesion regressed very rapidly, did the buds actually penetrate the caseous material for any distance. Usually the sprouts grew up to the epithelioid cells and sometimes between them, but were unable to enter the caseous area. The cells at the tip of the sprout apparently continued to divide so that the tip turned aside, dilated, and became bulbous. Capillary sprouts with dilated tips usually disappeared or went into stasis in a day or two. If healing was very slow, few capillary buds formed, and those which appeared were soon obliterated.

Fibrosis.—Fibrosis was not a marked feature of any of the lesions studied, except in one case where the tubercle had been present for over twelve months. Occasionally, when a tubercle was expanding very slowly, the collagen fibres which bordered the lesion became compressed, and a pseudo-capsule was formed.

Calcification.—Calcification was not seen in any of the ear-chamber tubercles, but was prominent in the caseous matter in sections from the rabbits with miliary tubercles, particularly in the lungs.

Histology of relapse.—When treatment ceased to be effective, the lesion started to spread, sometimes abruptly. The vessels which had grown into the cellular area around the caseous material went into stasis, and the tissue immediately adjoining the tubercle became necrotic. If the transition from healing to spread took place more slowly it was heralded by an increase in the number of polymorphonuclear leucocytes around the lesion, the margin of which became ill-defined.

DISCUSSION.

These experiments show that streptomycin and isoniazid, alone or together, could not completely destroy tubercle bacilli of the virulent Branch strain in the tissues of rabbits. On several occasions tubercles in the ear chamber resolved so completely that the site of the lesion was no longer distinguishable microscopically. Nevertheless, lesions reappeared at the same and at new sites when therapy was stopped, even if it had been maintained for as long as two months after apparent healing. When caseation had occurred healing was usually slow and incomplete, although on a number of occasions caseous material was absorbed.

The difficulty of healing a caseous lesion does not seem to be associated with the physical or chemical nature of caseous material itself. At first it was thought that some chemical constituent of caseous material inhibited vascular ingrowth, or that its semi-fluid nature did not give adequate support for the ingrowing capillaries. However, in the one case in which rapid absorption of caseous material did occur, there was prompt penetration of the caseous material by new capillary sprouts. This suggests that ingrowing capillaries are deflected from areas of caseation only when these contain living tubercle bacilli.

There is no doubt that in these experiments the animals were subjected to a very severe test, since the bacterium used was one to which the rabbit is highly susceptible.

Two points perhaps stand out when the bearing of these results on successful chemotherapy in man is considered : firstly, the need for earlier diagnosis than is now attainable, and secondly, the need for antituberculous drugs to be given for a much longer time *after healing appears to be complete* than is now done.

SUMMARY.

The effects of streptomycin, isoniazid, and the two drugs together have been observed on growing tubercles in transparent chambers in the rabbit's ear.

Streptomycin and isoniazid alone or together could not completely destroy tubercle bacilli of the " Branch " strain.

Non-caseous lesions were seen to disappear but reappeared when treatment was withdrawn. Caseous lesions healed incompletely and slowly. The inability of

capillaries readily to penetrate caseous material may be associated with the presence of live tubercle bacilli.

A description is given of the cytological changes to be seen in a healing tubercle and a comparison is made with the lesions of miliary tubercles in the lung and liver.

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PATHOGENESIS OF CORTISONE-INDUCED EXOPHTHALMOS IN GUINEA-PIGS.

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EXOPHTHALMOS has been reported to occur in various animals injected with cortisone acetate : in rats (Aterman and Greenberg, 1953), in guinea-pigs (Aterman, 1951 ; 1952), and in rats, guinea-pigs and rabbits (Wynn Williams, 1953). Boas and Scow (1954) investigated the effect of cortisone on the size of the skull, the orbit and the orbital contents in young rats and concluded that the exophthalmos was due to a marked inhibition of head growth in relation to a normal growth of the eye-ball. The present experiments were designed to investigate the possibility of a similar mechanism of production in the cortisone-treated guinea-pig. The animals were given a normal mixed diet and plenty of water to drink.

EXPERIMENTAL.

Experiment 1.—Fourteen young female guinea-pigs were given a daily intramuscular injection of cortisone acetate (Merck), the dose being 5 mg./100 g. body wt., while 14 control animals received the same volume of cortisone suspending medium. Both groups were matched for weight at the beginning of the experiment. The average initial weight was 310 g. and 303 g. for the test and control groups respectively. The inter-corneal distance was carefully measured with a pair of dividers (Wynn Williams, 1953) at the beginning and end of the experiment which lasted 15 days. The weights of the animals were also taken at the end of the experiment. The animals were killed with chloroform. After death, the eyeball was removed and its volume measured by water-displacement ; using a detergent solution (" Teepol ") and a tuberculin syringe with a No. 1 gauge needle the volume of the eye socket was also measured. The retro-bulbar tissues (Harderian glands, fibro-fatty connective tissue, extrinsic ocular muscles and optic nerve) were then removed, finely minced and their volume determined. The head was then cut off at the foramen magnum and the soft tissues of the skull were easily removed after autoclaving at a pressure of 15 lb. per sq. in. (1 kg./sq. cm.) for 15 min. The dried skulls were weighed and the volume of the empty orbit determined, again using the detergent solution and syringe after the foramina had been sealed with a minimum of clay, care being taken to treat test and control skulls in an identical manner. Finally, the length and various breadths (Figure) of the dry skull were measured with dividers.

No significant difference was found in the volume of the eyeball in the two groups. The other results, which are set out in the Table, showed that in the test group there is a slight though not significant gain in weight ; the inter-corneal distance is significantly greater in the test group, while in all other measurements

the test group shows average values significantly smaller than in the control group. These findings indicate that cortisone retards the growth of the bony skull and the retro-orbital fat in the growing guinea-pig. In *long*-term experiments, *e.g.*, 3 months, the weight and general body size of cortisone-treated guinea-pigs are

FIGURE.—Skull measurements: A = length, B = frontal breadth, C = malar breadth, D = occipital breadth

TABLE.—*Skull Measurements in Cortisone-treated and Control Guinea-pigs.*

All averages based on 14 animals in each group.

Measurement.	Unit.	Cortisone. Average ± S.E.	Control. Average ± S.E.	Significance levels for differences between averages.
Weight gain	g.	68·2 ±5·6	56·8 ±6·5	Not significant
Inter-corneal distance (increase)	cm.	0·24±0·01	0·08±0·01	0·1 per cent
Volume of bony orbit	ml.	0·51±0·02	0·61±0·02	0·1 „ „
Volume of eye socket	ml.	0·16±0·01	0·20±0·01	2·5 „ „
Volume of orbital fat	ml.	0·26±0·01	0·33±0·02	0·1 „ „
Weight of dry skull	g.	4·6 ±0·1	5·1 ±0·1	1 „ „
Skull length	mm.	54·5 ±0·5	56·9 ±0·4	1 „ „
Occipital breadth	mm.	20·9 ±0·2	21·9 ±0·1	0·1 „ „
Frontal breadth	mm.	11·8 ±0·2	12·4 ±0·2	2·5 „ „
Malar breadth	mm.	30·4 ±0·3	31·4 ±0·2	1 „ „

conspicuously less than those of control litter-mates, but in the present short-term experiments the weight of the test animals was if anything greater than in the control animals; this tendency to increased weight is probably due to water retention. It appears that the exophthalmos is at least partly the result of restricted accommodation for the eyeball.

Experiment 2.—Six fully mature female guinea-pigs (average weight 1100 g.) were given daily intramuscular injections of cortisone acetate, 5 mg./100 g. body wt. for 15 days. These animals had been weighed daily for 6 weeks before the beginning of the experiment and had shown no significant gain or loss in weight. Their inter-corneal distance and body weight were determined on the first and last days of the experiment. The results showed no alteration in either of these measurements. The conclusion is therefore drawn that cortisone produces exophthalmos only in the *growing* guinea-pig.

DISCUSSION.

The exophthalmos produced in young rats injected with cortisone for 40 days was attributed by Boas and Scow to an increase in the ratio of protruding eyeball mass to head size. This disproportion resulted primarily from retarded orbital growth without any retardation in growth of the eyeball. A similar disproportion between eyeball and body growth has also been observed in young thyroidectomised guinea-pigs (Boas, 1953, unpublished observations) and in cortisone-treated chick embryos (Evans, 1953). By calculation from their experimental data, Boas and Scow found that there was no abnormal displacement of the eyeballs from the orbit and they therefore defined the exophthalmos in the rat as "apparent". They omitted, however, to measure the inter-corneal distance. Clinically, exophthalmos in man is associated with an increase in the mass of retrobulbar tissues (Dobyns, 1950) and experimental exophthalmos produced with pituitary preparations in guinea-pigs is also associated with an increase in the mass of retrobulbar tissues (Smelser, 1937). The results of the present experiment show that exophthalmos produced by injecting cortisone in the growing guinea-pig is related largely to retarded growth of the skull and is not accompanied by any increase in the mass of retrobulbar tissues.

SUMMARY.

Exophthalmos occurs in growing guinea-pigs but not in fully mature guinea-pigs injected with cortisone.

The exophthalmos appears to be largely the result of retarded growth of the skull, resulting in a disproportion between the volume of eyeball and orbit.

I am grateful to the Medical Research Council for supplies of cortisone, to Mr. W. Z. Billerwicz for statistical help, and to Professor J. S. Young for encouragement and advice.

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THE EFFECT OF CERTAIN FATTY ACIDS ON THE COAGULATION OF PLASMA *IN VITRO*.

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FULLERTON, DAVIE AND ANASTASOPOULOS (1953) showed that following a meal consisting of bacon and eggs, bread and butter, there occurs a significant shortening of the whole blood clotting time in siliconed tubes and of the accelerated plasma clotting time using Russell's viper venom. These results have been confirmed and extended by O'Brien (1955) who showed that the calcium time of plasma is similarly shortened following a fatty meal. Macfarlane, Trevan and Attwood (1941) found that plasma freed from particulate fat by high speed centrifuging showed a considerable prolongation of its clotting time in the presence of Russell's viper venom : the defect could be corrected by replacing the fatty supernatant material removed in the centrifuging process. Thus it seemed reasonable to postulate that the acceleration of coagulation which follows fat feeding might be due to increased numbers of circulating chylomicra. However, in a previous paper (Poole, 1955) it was shown that while it was possible to shorten the calcium time of plasma by adding a washed chylomicron suspension, it was necessary, in order to produce changes comparable with those following fat feeding, to add chylomicra in a concentration greatly in excess of anything which is likely to occur in physiological lipæmia.

The object of this paper is to examine the possibility that free fatty acids contribute to the changes in blood coagulability following fat feeding, by studying the effects of the addition of fatty acids to plasma *in vitro*.

METHODS.

Plasma.—Fresh human plasma has been used. Blood was collected into 3·8 per cent trisodium citrate solution in the proportion of 9 parts of blood to 1 part of citrate solution and the plasma was separated by centrifuging at 4000 rev./min. for 15 min.

Fatty acids.—Experiments have been carried out with the sodium salts of fatty acids. These have been used either as fine aqueous suspensions (prepared by heating the substance with water until it dissolves and then allowing the solution to cool) or as solutions in 5 per cent albumin. Such albumin solutions possibly represent the form in which free fatty acids appear in the circulation under physiological conditions.

Calcium time.—To 0·1 ml. of citrated plasma was added 0·1 ml. of the solution whose activity was being tested in a 63 × 9 mm. test tube standing in a 37° water bath ; 0·1 ml. of 0·025 M calcium chloride solution was then added and the time taken for the mixture to clot was recorded.

Russell's viper venom accelerated clotting time.—To 0·1 ml. of citrated plasma were added 0·1 ml. of the solution whose activity was being tested and 0·1 ml. of 1/10,000 Russell's viper venom (" Stypven ", Burroughs Wellcome) in a 63 × 9 mm. test tube in a 37° water bath ; 0·1 ml. of 0·025 M calcium chloride solution was then added and the time taken for the mixture to clot was recorded.

Thrombin generation test.—The technique of Pitney and Dacie (1953) has been followed.

RESULTS.

A Time-consuming Reaction between Fatty Acids and Plasma.

When a concentration of 3·3 m-equiv./l. of the sodium salt of certain fatty acids is added to plasma, a shortening of the calcium time occurs, to an extent which depends on the time which has elapsed between mixing the plasma with the fatty acid sodium salt and adding the calcium chloride solution. Qualitatively and quantitatively similar results were obtained with aqueous suspensions and

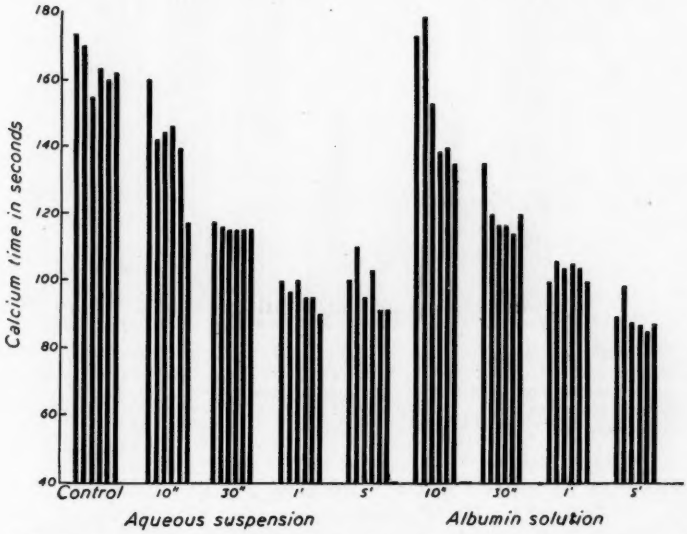

FIG. 1.—Effects of sodium stearate 3·3 m-equiv./l. on the calcium time of normal plasma. To 0·1 ml. citrated plasma was added 0·1 ml. of an aqueous suspension or an albumin solution of sodium stearate. The mixture was recalcified with 0·1 ml. 0·025 M-$CaCl_2$ after pre-incubation at 37° for varying periods of time. The columns represent individual calcium time determinations. Six replicate determinations were made for each set of conditions.

with albumin solutions. Fig. 1 shows the results obtained with sodium stearate. A maximum effect is reached in about 1 min.

Qualitative Differences between Different Fatty Acid Sodium Salts.

Fig. 2 shows the effects obtained with aqueous suspensions of a selection of sodium salts of long chain fatty acids at a concentration of 3·3 m-equiv./l. In each case the suspension was mixed with the plasma 1 min. before recalcification. Stearate, palmitate and oleate produced a substantial acceleration of coagulation, whereas elaidate, linoleate and ricinoleate had no measurable effect. The substances used were described as pure by the manufacturers, but in view of the very considerable difficulties which are encountered in purifying fatty acids, it is not possible to exclude the presence of small amounts of other fatty acids in these

preparations. The following acids have also been tested in a similar manner and have shown no effect on the calcium time: acetic, butyric, valeric, caproic, pelargonic, caprylic, lauric, myristic.

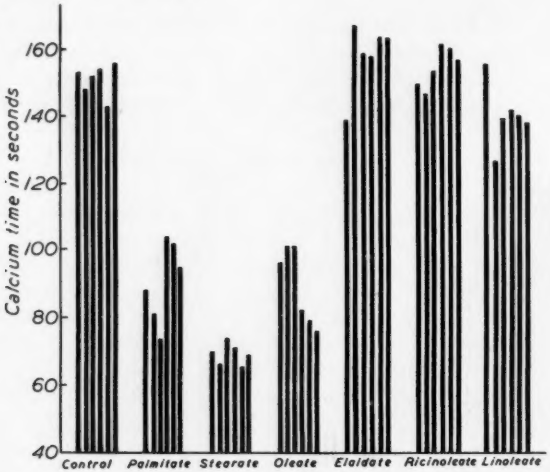

FIG. 2.—Effects of aqueous suspensions of various fatty acid sodium salts (3·3 m-equiv./l.) on the calcium time of normal plasma. A mixture of 0·1 ml. citrated plasma with 0·1 ml. of an aqueous suspension of the fatty acid sodium salt was incubated at 37° for 1 min. and then recalcified with 0·1 ml. 0·025 M–$CaCl_2$. The columns represent individual calcium time determinations.

The Concentration in which Certain Fatty Acid Sodium Salts Accelerate Plasma Coagulation.

A maximal effect is obtained with a concentration of about 3 m-equiv./l. Substantially higher concentrations have an anticoagulant effect. The titration of the effect of sodium stearate given in Table I shows that even with a concentration of 20 μ-equiv./l. a significant effect can be demonstrated.

pH Changes produced by the Addition of Fatty Acids to Plasma.

Stearic acid or sodium stearate added to plasma in a concentration of 3·3 m-equiv./l. produce no change in pH which can be detected with a pH meter. The effect on coagulation is not measurably different whether the test is carried out with the fatty acid, its sodium salt, or the sodium salt to which so much hydrochloric acid has been added as may be necessary to produce neutral pH. It seems, therefore, that pH changes cannot be responsible for the effects described.

Effects of Sodium Salts of Fatty Acids on the Russell's Viper Venom Accelerated Clotting Time.

The sodium salts of certain fatty acids in aqueous suspension at a concentration of 3·3 m-equiv./l. shorten the Russell's viper venom accelerated clotting time of plasma but the results obtained are qualitatively different from the results of

TABLE I.—*Titration of Accelerating Effect of Sodium Stearate on the Calcium Time of Normal Plasma.*

Concentration (m-equiv./l.).	Clotting time (sec.).
0	147, 157
0·02	123, 127
0·04	119, 124
0·06	105, 122
0·08	100, 118
0·10	97, 105
0·20	92, 90
0·40	86, 87
0·60	82, 85
0·80	77, 82
1·0	73, 80
2·0	83, 69
3·0	79, 76
4·0	77, 71
5·0	89, 89

Plasma 0·1 ml. + 0·1 ml. stearate suspension incubated at 37° for 60 sec. and then recalcified with 0·1 ml. 0·025 M–$CaCl_2$. Two replicate determinations at each concentration.

calcium time determinations. With sodium stearate, a time-consuming reaction again occurs: no acceleration is observed when plasma, stearate, venom and calcium chloride solution are mixed as rapidly as possible; if, however, the plasma-stearate-venom mixture is incubated for a short time before recalcification the clotting time is shortened. However, in addition to this delayed acceleration, an immediate acceleration is obtained with sodium laurate and to a lesser extent with sodium oleate and linoleate. Experimental results illustrating these changes are shown in Table II.

TABLE II.—*Immediate Effects of Sodium Salts of Fatty Acids on Clotting Times Accelerated by Russell's Viper Venom.*

Fatty acid.	Clotting time (sec.).							
Control	16	17	18	17	19	18	17	18
Lauric	10	12	12	11	9	11	10	12
Myristic	17	18	17	18	17	17	17	17
Palmitic	16	17	18	16	16	16	16	16
Stearic	19	18	17	18	17	19	16	18
Oleic	15	14	11	14	14	14	14	14
Elaidic	17	17	17	19	18	19	17	17
Linoleic	14	14	13	15	15	16	14	14

Plasma 0·1 ml. + 0·1 ml. 1/10,000 Russell's viper venom + 0·1 ml. Na-fatty acid suspension (final concn. 3·3 m-equiv./l.) mixed together and recalcified immediately with 0·025 M–$CaCl_2$. Eight replicate determinations for each substance tested.

Effects of Fatty Acid Sodium Salts on Thrombin Generation.

O'Brien (1955) has shown that in human subjects after fat feeding the Russell's viper venom accelerated clotting times are closely correlated with the minimum fibrinogen times obtained in the thrombin generation test, using a technique resembling the modification by Pitney and Dacie (1953) of the procedure introduced by Macfarlane and Biggs (1953). In view of O'Brien's results it was of interest to see whether the effect of sodium laurate on the Russell's viper venom accelerated

clotting time of plasma is paralleled by a shortening in the fibrinogen times recorded in the thrombin generation test. The experiments given in Fig. 3 and 4 show that this is the case : sodium stearate increases the amount of thrombin produced and shortens the time required to reach a maximum concentration of thrombin ; sodium laurate on the other hand, increases the peak concentration of

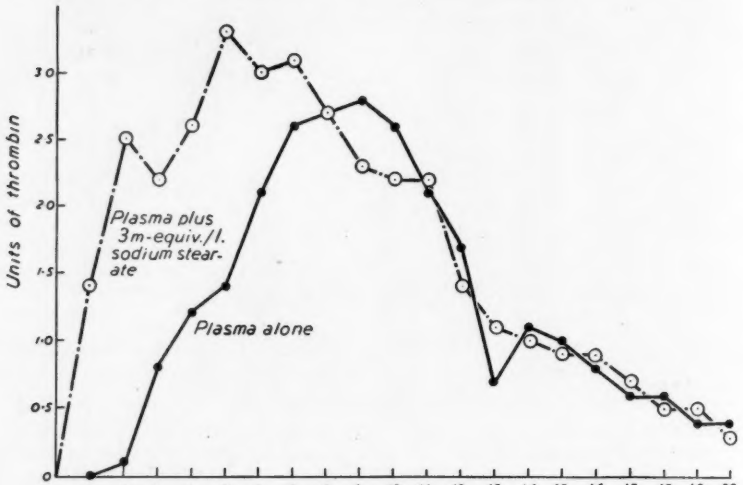

FIG. 3.

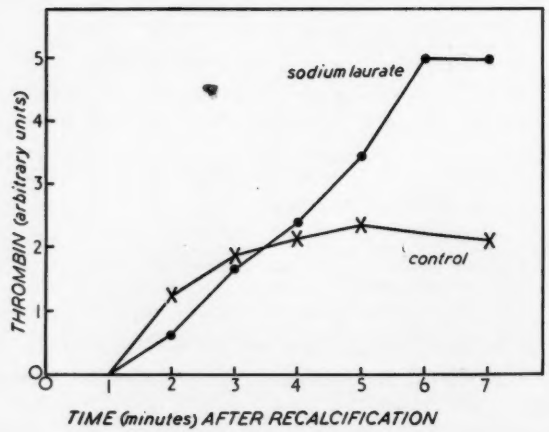

FIG. 4.

FIG. 3 and 4.—Thrombin generation from mixtures of equal vol. of citrated plasma, 3·3 m-equiv./l. fatty acid sodium salt suspension and 0·025 M–$CaCl_2$. The plasma and fatty acid sodium salt suspension were incubated together at 37° for 1 min. before recalcification.

thrombin but does not shorten the time required to reach a maximum concentration.

DISCUSSION.

French, Robinson and Florey (1953) showed that plasma from a rat which had received an injection of heparin would clear added chyle. Robinson and French (1953) found that the clearing phenomenon *in vitro* was accompanied by the conversion of triglycerides into free fatty acid attached to the plasma albumin. Jeffries (1954) demonstrated a similar but weaker clearing activity in the plasma of rats after olive oil feeding, the effect being considerably enhanced by ligating the animal's thoracic duct shortly before exsanguination. Robinson, Jeffries and French (1954) found that the clearing activity produced by fat feeding closely resembles that which follows heparin injection. Hence it is probable that the ingestion of fat is followed by the appearance of free fatty acids in the blood; this supposition receives confirmation from the work of Grossman, Palm, Becker and Moeller (1954) who found that the plasma of fat-fed rats had approximately twice the free fatty acid concentration found in the plasma of starved rats :· the increase in concentration was of the order of 0·3 m-equiv./l. Direct evidence of clearing activity in man following fat feeding is so far lacking, but Jeffries (unpublished observations) has demonstrated such activity in a number of other mammalian species and it seems likely that a similar chemical reaction, leading to the production of circulating free fatty acid, occurs in man. If the appearance of free fatty acid *in vivo* has the same effect on coagulation as can be demonstrated by the addition of fatty acids to plasma *in vitro*, then the increase in concentration reported by Grossman *et al.* (1954) would be more than sufficient to account for the increased coagulability of blood produced by fat feeding.

SUMMARY.

Certain fatty acids, in low concentration, appear to accelerate the coagulation time of recalcified citrated human plasma.

I thank Professor Sir Howard Florey, F.R.S. and Dr. R. G. Macfarlane for constant advice and encouragement, my colleagues Drs. J. E. French, G. H. Jeffries and D. S. Robinson for valuable suggestions, and Dr. J. C. Smith, who kindly supplied some of the fatty acids tested.

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CHEMICAL CONSTITUTION AND ANTI-TUBERCULOUS ACTIVITY: PART II: BASES POSSESSING THE DIPHENYL STRUCTURE.

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IN a previous publication in this series (Cymerman-Craig, Rubbo and Pierson, 1954) 45 primary aromatic amines were investigated. It was shown that activity was proportional to the magnitude of the total planar surface area, and thus appeared to be primarily a physical (*i.e.*, a generalised adsorption) phenomenon.

The aim of the present investigation has been to find, in a series of compounds of *constant* surface area, the physical and chemical basis for anti-tuberculous activity. To this end 46 basic diphenyl compounds (I) were examined. The results show a correlation between activities, basic strengths and lipoid/water partition coefficients, and indicate certain minimal requirements for optimum anti-tuberculous activity in this series.

MATERIALS AND METHODS.

Organic chemistry.—The compounds listed in Tables I and II were synthesised for this work to provide substances having the desired physical properties. Their preparation has been described by Bauer and Cymerman (1950*a*, *b*), Bauer, Cymerman and Sheldon (1951), and Baxter, Cymerman-Craig and Gilbert (1953). All substances tested were of analytical purity.

Physical chemistry.—The basic strengths and partition coefficients quoted in this paper were determined by Carswell, Cymerman and Lyons (1952), Baxter *et al.* (1953), Allen, Cymerman-Craig and Diamantis (1954), Cymerman-Craig and Diamantis (1953), and Cymerman-Craig, Parker and Woodhouse (1953).

The percentage of these compounds ionised at pH 7·2 in water has been calculated as previously described (Cymerman-Craig *et al.*, 1954) and those pKa values obtained by adding a correction to the value found in 50 per cent aqueous alcohol are placed in parentheses in the Tables. Partition coefficients were determined between liquid paraffin (B.P. 1932) and water at pH 7·2.

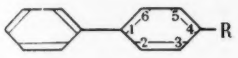
Bacteriological.—The tuberculostatic tests were performed by the serial dilution technique using *Mycobacterium tuberculosis* H37Rv as previously described (Cymerman-Craig *et al.*, 1954), the incubation period being fixed at 14 days at 37° in paraffin-sealed tubes. Bacteriostatic tests with *Staphylococcus aureus*, *Bacterium coli* and *Pseudomonas pyocyanea* were carried out in beef extract peptone water (pH 7·2–7·4) as described by Rubbo, Albert and Maxwell (1942).

RESULTS.

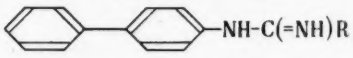
N-4-Diphenylylamidines.—In Table I*a* the examination of 10 N-4-diphenylylamidines (II) is recorded. These are more than 95 per cent ionised at pH 7·2, and lipoid/water partition coefficients increase regularly with the size of the lipophilic group from 0·18 to > 100. Anti-tuberculous activity increases to a

maximum value of M/16,000–M/32,000 in serum synthetic medium, but these values are usually considerably higher in the non-protein synthetic medium.

p-Phenylbenzamidines.—Table I*b* contains the activities of 9 *p*-phenylbenzamidines (III), including a cyclic analogue (IV), for which the pKa varies from 8 to 11; these compounds are also 90–100 per cent ionised at the physiological pH, and lipoid/water partition coefficients range from 0·075 to 7·5, a 100-fold range. A further cyclic analogue, No. 104 (V), is a much weaker base, un-ionised at pH 7·2, and could not be tested in stronger than M/8,000 solution



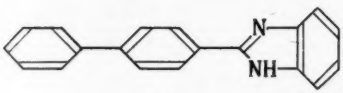
(I)



(II)

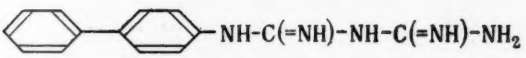
(III)

(IV)



(V)

(VI)



(VII)

This group of substances was prepared to test the suggestion (Erlenmeyer, Becker, Sorkin, Bloch and Suter, 1947) that a partial *p*-aminobenzoic acid structure (VI) is essential for, or enhances, anti-tuberculous activity. Compounds of type (II) fall into this category, whereas those of type (III), while isomeric with the former and similar in all physical and chemical respects, do not possess the *p*-aminobenzoic acid skeleton. Table I*b* shows that in this series also, activity increases to a maximum of M/16,000 and that there is no significant difference between isomeric compounds in the two series (II) and (III).

One other factor emerges from Table I. It is readily seen that maximum activity is not reached until the partition coefficient exceeds a value of about 0·2, and that activity is not enhanced as the lipophilic properties of the compounds increase beyond this ratio (Fig. 2).

N-Substituted 4-aminodiphenyls.—By introduction of a variety of nitrogenous substituents R in (I), it has been possible to vary both the basic strengths and the lipoid/water partition ratios. Table II*a* and *b* contains 27 diphenyl derivatives varying in basic strength from a pKa of 2·35 to one of 10·96, and partition

TABLE I.—(a) *N*-4-*Diphenylylamidines and* (b) p-*Phenylbenzamidines.*

Highest dilution expressed as 1/molarity of the substance completely inhibiting growth.

Lab. ref. number.	Substance. (*a*)	Structure (II); R =	Organisms and media: H37Rv serum-synthetic medium.	H37Rv synthetic medium.	*Staph. aureus* (broth).	*Bact. coli* (broth).	pKa in water at 20°.	Percentage ionised pH 7·2.	Lipoid/water partition coefficient pH 7·2.
3	N-4-Diphenylylacetamidine	Me	2000	4000	<1000	<1000	(9·66)	99·7	0·18
4	1-4′-diphenylylamidino-*n*-pentane	C_5H_{11}	16000	128000	4000	4000	(9·66)	99·7	1·9
5	1-4′-diphenylylamidino-*n*-heptane	C_7H_{15}	32060	128000	32000	<8000	(9·54)	99·5	26·0
9	1-4′-diphenylylamidino-*n*-hexadecane	$C_{16}H_{33}$	16000	64000	<8000	,,	(9·57)	99·6	> 100
27	1-4′-diphenylylamidino-cyclopentene-1	C_5H_7	32000	32000	2000	<2000	(9·06)	98·6	3·4
6	1-4′-diphenylylamidino-cyclohexene-1	C_6H_9	16000	64000	4000	,,	(9·25)	99·1	7·5
38	1-4′-diphenylylamidino-cyclohexane	C_6H_{11}	8000	16000	2000	,,	(9·60)	99·6	7·6
10	N-4-diphenylylbenzamidine	Ph	16000	64000	<8000	<8000	(8·50)	95·2	7·0
7	*p*-ethoxy-N-4-diphenylylbenzamidine	C_6H_4OEt	<16000	<16000	,,	,,	(8·65)	96·6	19·0
29	3:4-dimethoxy-N-4-diphenylylbenzamidine	$C_6H_3(OMe)_2$	8000	8000	,,	,,	(8·61)	96·5	33·0
	(*b*)	(III); R =							
2	*p*-Phenylbenzamidine	H	2000	2000	2000	2000	(11·49)	100	0·075
39	*p*-phenyl-N-*n*-butylbenzamidine	C_4H_9	4000	8000	2000	<2000	(11·13)	100	0·20
49	N-cyclohexyl-*p*-phenylbenzamidine	C_6H_{11}	16000	16000	4000	,,	(11·16)	100	0·21
46	N-phenyl-*p*-phenylbenzamidine	Ph	16000	16000	<8000	<8000	(8·35)	93·3	6·4
11	*p*-phenyl-N-*p*-ethoxyphenylbenzamidine	C_6H_4OEt	16000	32000	,,	,,	(8·52)	95·2	6·7
68	*p*-phenyl-N-*p*-chlorophenylbenzamidine	C_6H_4Cl	8000	8000	,,	,,	(8·14)	89·9	—
69	*p*-phenyl-N-*p*-piperidinophenylbenzamidine	$C_6H_4NC_5H_{10}$	16000	128000	,,	,,	(8·40)	94·1	7·5
59	2-4′-diphenylyl-4:5-dihydroglyoxaline	(IV)	4000	4000	2000	<2000	(9·69)	99·7	0·11
104	2-4′-diphenylylbenzaminazole	(V)	<8000	<8000	<8000	<8000	(4·04)	0·07	—

TABLE II.—(a) *Substituted 4-Aminodiphenyls and* (b) *Other Diphenyl Compounds.*

Highest dilution expressed as 1/molarity of the substance completely inhibiting growth.

Lab. ref. number.	Substance (a)	Structure (I); R =	Organisms and media: H37Rv serum-synthetic medium.	H37Rv synthetic medium.	*Staph. aureus* (broth).	*Bact. coli* (broth).	pKa in water at 20°	Percentage ionised at pH 7·2.	Lipoid/water partition coefficient pH 7·2.
1	4-Aminodiphenyl	NH_2	2000	4000	2000	<1000	4·34	0·14	55·0
117	N-4-diphenylylethylamine	NH Et	<8000	16000	<8000	<8000	(4·92)	0·5	1550
20	4-dimethylaminodiphenyl	NMe_2	„	„	„	„	(4·87)	0·47	92
31	4-diphenylyl trimethylammonium iodide	NMe_3I	<2000	2000	<2000	<2000	10	99·9	0·04
82	4-formamidodiphenyl	NHCHO	<4000	8000	<8000	<8000	(2·45)	0·061	—
137	4-acetamidodiphenyl	$NHCOCH_3$	2000	2000	<2000	<2000	(2·35)	0·001	—
74	4-benzylideneaminodiphenyl	N:CH Ph	8000	8000	<8000	<8000	—	—	—
75	4-*p*-methoxybenzylideneaminodiphenyl	N:CH-C_6H_4OMe	<8000	„	„	„	(4·45)	0·18	—
40	4-*p*-hydroxybenzylideneaminodiphenyl	N : CH-C_6H_4OH	4000	„	„	„	—	—	—
41	4-*p*-dimethylaminobenzylideneaminodiphenyl	N:CH-$C_6H_4NMe_2$	<8000	„	„	„	—	—	—
105	N-di-2′-hydroxyethyl-4-aminodiphenyl	$N(CH_2CH_2OH)_2$	4000	4000	<4000	<4000	(3·60)	0·03	0·26
106	3-N-4′-diphenylylaminopropan-1:2-diol	$NHCH_2CHOH\ CH_2OH$	2000	4000	<2000	<2000	(3·74)	0·03	0·08
21	N′-4′-diphenylyl-N:N-diethylethylenediamine	NH $CH_2CH_2NEt_2$	8000	16000	1000	<1000	(8·70)	97·0	44·7
67	N′-4′-diphenylyl-N:N-diethyl-N-N′-N′-trimethylethylenediammonium di-iodide	$NMe_2CH_2CH_2$ NEt_2Me:2I	<2000	8000	<2000	<2000	12	.100	0·065
76	4-benzylaminodiphenyl	NH CH_2Ph	8000	16000	<8000	<8000	—	—	—
77	4-*p*-hydroxybenzylaminodiphenyl	NH $CH_2C_6H_4OH$	4000	8000	<4000	<4000	—	—	—
78	4-*p*-dimethylaminobenzylaminodiphenyl	NH $CH_2C_6H_4NMe_2$	8000	16000	<8000	<8000	—	—	45·0
79	4-*p*-methoxybenzylaminodiphenyl	NH $CH_2C_6H_4OMe$	2000	8000	<2000	<2000	(3·97)	0·06	—
85	N-4-diphenylyl-2-morpholinoethylamine	NH $CH_2CH_2NC_4H_8O$	4000	„	„	„	(6·43)	14·0	74·9
86	N-4-diphenylyl-N:N-dimethyl-2-N′-methylmorpholinoethylammonium di-iodide	$NMe_2CH_2CH_2$ $NMeC_4H_8O.2I$	<2000	„	<2000	„	11	.100	0·16
84	N-4-diphenylylguanidine	NH C(=NH) NH_2	1000	2000	2000	<2000	(10·96)	.100	0·10
96	N-4-diphenylylbiguanide	NHC(=NH)NHC (=NH)NH_2	64000	128000	4000	„	(11·0)	.100	0·60
	(b)								
43	2-aminodiphenyl	—	<2000	2000	<2000	<2000	3·84	0·04	126
42	3-aminodiphenyl	—	1000	„	2000	<1000	(4·37)	0·15	47
44	3-amino-2′-methoxydiphenyl	—	1000	„	1000	„	—	—	—
		(I); R =							
22	4-2′-diethylaminoethoxydiphenyl	$OCH_2CH_2NEt_2$	8000	16000	1000	1000	(8·70)	97·0	104
23	4-diphenylyloxyethyldiethylmethylammonium iodide	$OCH_2CH_2NEt_2Me$ .I	2000	4000	<2000	<1000	12	.100	0·12

coefficients ranging from 0·04 to 1550, a 40,000-fold variation. No. 40, 41, 74, 76, 77 and 78, like their analogues No. 75 and 79, would be non-ionised at pH 7·2.

Activities do not rise beyond a maximum value of M/8000 in presence of serum. It will be noted that a number of compounds which are fully ionised and yet show low activity (*e.g.*, No. 31, 67, 86, 84 and 23) have in common the possession of a very small partition coefficient, of magnitude less than 0·2 (Fig. 2).

The activities of the diphenyl derivatives in Tables I and II against *Staph. aureus* and *Bact. coli* are uniformly low, exceeding M/4000 in only one case (No. 5). *Ps. pyocyanea*, not recorded in the Tables, grew in all concentrations.

DISCUSSION.

The two factors which have been investigated are variation in (1) basic strength, and (2) lipoid/water partition coefficient, for a series of diphenyl derivatives (I) which exhibit a wide variation in these two properties and yet possess an approximately constant *flat* surface area.

The effect of change in basic strength is shown in Fig. 1, in which the anti-tuberculous activity of the bases is plotted against pKa units. Activity is

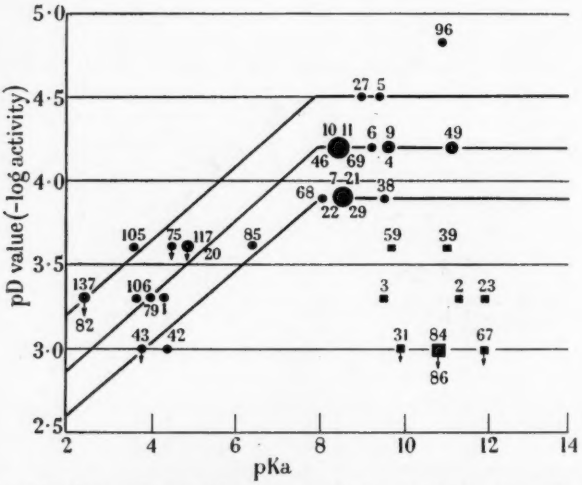


FIG. 1.—The relationship of anti-tuberculous activity to basic strength. ■ denotes lipoid/water partition coefficient less than 0·2.

signifies that the tuberculostatic end-point lies at or below the level indicated.

expressed in log dilutions, or pD values, where the pD value is the negative logarithm of the lowest tuberculostatic molar concentration completely inhibiting growth in presence of 10 per cent of serum at pH 7·2 as described previously (Cymerman-Craig *et al.*, 1954). The points represented by the individual compounds are at the centre of the circle or square adjoining each number. It is

seen that most of the points fall near the curve drawn through them. This curve has been expanded into a shaded area 0·3 unit above and below the mean values to allow for the twofold variation inherent in the bacteriostatic end-point. Only a few points lie outside this shaded area. Those lying below the curve are No. 59, 39, 3, 2, 23, 31, 84, 86 and 67 and *each* of these highly-ionised substances possesses a partition coefficient *less than* 0·2 (indicated by a square on the Figure).

From Fig. 1 there appears to be a direct correlation between anti-tuberculous activity and basic strength, optimum activity being attained only by those members possessing a pKa greater than 8·0, *i.e.*, at least 85 per cent ionisation at pH 7·2. Further increase in the pKa does not produce a corresponding enhancement in activity.

One substance (No. 96), N-4-diphenylylbiguanide (VII), shows activity considerably higher than other members of this series. Biguanides form metal complexes, and No. 96 is the only member of this series capable of such chelation, which may account for its elevated activity.

The relation of anti-tuberculous activity to lipoid/water partition coefficient is shown in Fig. 2, where the activity at pH 7·2 is plotted against the partition

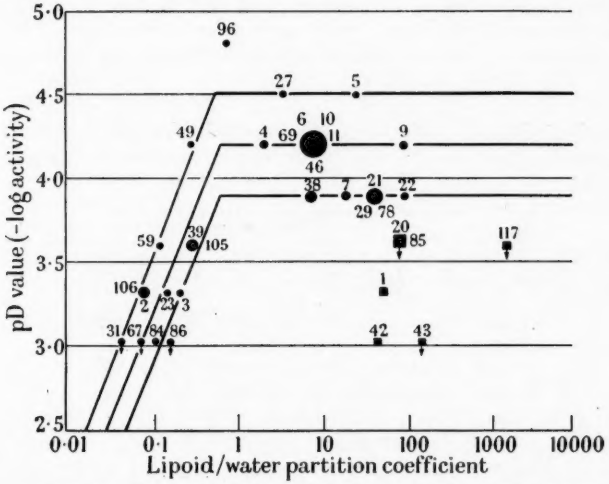


FIG. 2.—The relationship of anti-tuberculous activity to lipoid/water partition coefficient. ■ denotes pKa less than 8·0.

signifies that the tuberculostatic end-point lies at or below the level indicated.

coefficient at the same pH. A curve resembling that of Fig. 1 can be drawn through the experimental points, and when expanded, as in Fig. 1, only a few points fall outside the shaded portion. These are No. 42, 43, 1, 117, 20 and 85. Inspection of Tables I and II shows that all these substances are weak bases, *i.e.*, have a pKa less than 8·0.

These results indicate two simultaneous physico-chemical requirements for optimum activity in this series. The first is high ionisation (pKa greater than 8·0), and the second an adequate partition coefficient (in excess of about 0·2). Further, neither an increase in ionisation nor an increase in partition coefficient beyond 0·2 has any effect on activity. Hence, the *absolute* lipoid-solubility *per se* does not appear to be an important factor. Ionisation may facilitate union of nitrogenous cations with oppositely-charged receptor groups on the organism and limited lipophilic character may favour an economic phase transfer of the drug across the bacterial surface.

SUMMARY.

Forty-six diphenyl compounds have been examined for their anti-tuberculous activity.

Anti-tuberculous activity has been found to require a pKa of not less than 8·0, corresponding to at least 85 per cent ionisation at pH 7·2. Higher pKa values do not enhance activity.

Anti-tuberculous activity is also related to the lipoid/water partition coefficient requiring a partition coefficient of not less than about 0·2. Increased values do not result in greater activity.

Optimal activity is shown only by compounds having both these physical factors.

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CHEMICAL CONSTITUTION AND ANTI-TUBERCULOUS ACTIVITY: PART III: HETEROCYCLIC BASES.

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A CORRELATION between anti-tuberculous activity and molecular flat surface area has been demonstrated for a series of non-ionised primary aromatic amines (Cymerman-Craig, Rubbo and Pierson, 1954), and in a series of basic diphenyl compounds of approximately constant planar surface area it was found (see preceding paper) that anti-tuberculous activity depended on the degree of ionisation and lipoid/water partition coefficient. Thus it appeared that for maximum anti-tuberculous activity the simultaneous possession of three physical attributes would be necessary: (*a*) a molecule with a reasonably large planar surface area; (*b*) high degree of ionisation; and (*c*) a lipoid/water partition coefficient greater than 0·2 at pH 7·2. The aim of the present work has been to test this concept in a group of 27 selected heterocyclic aromatic amines, some of which fulfil all three requirements. It has been possible to show that there exists again a correlation firstly between activity and ionisation, and secondly between activity and molecular planar surface areas, and that a 2000-fold increase in activity can be achieved by making use of these criteria.

MATERIALS AND METHODS.

Organic chemistry.—The compounds in Tables I and II were synthesised for this work to provide substances having the desired physical properties. Some were previously unknown, and their preparation is described by Baxter and Cymerman-Craig (1953) and Cymerman-Craig and Loder (unpublished work). All substances tested were of analytical purity.

Physical chemistry.—The previously unknown basic strengths and lipoid/water partition coefficients quoted in this paper were determined by Cymerman-Craig and Diamantis (1953), Cymerman-Craig, Parker and Woodhouse (1953) and Cymerman-Craig and Loder (unpublished work). Those pKa values in water placed in parentheses in the Tables were obtained by adding a correction to the value determined in 50 per cent aqueous alcohol. For benzacridines this correction, obtained from pKa values determined both in water and alcohol, has the magnitude 0·7 unit, in excellent agreement with the value obtained by Albert, Rubbo and Burvill (1949). Partition coefficients were determined between liquid paraffin (B.P. 1932) and water at pH 7·2.

Bacteriological.—The techniques, test organisms and media (*Mycobacterium tuberculosis* H37 Rv, *Staphylococcus aureus*, *Bacterium coli* and *Pseudomonas pyocyanea*) were as described previously (Cymerman-Craig *et al.*, 1954; Rubbo, Albert and Maxwell, 1942); the tuberculostatic end-point was taken as that dilution completely inhibiting visible growth after 14 days' incubation at 37°.

TABLE I.—*Heterocyclic Bases.*

Highest dilution expressed as 1/molarity of the substance completely inhibiting growth.

Lab. ref. number.	Substance.	Organisms and media. H37Rv serum-synthetic medium.	H37Rv synthetic medium.	*Staph. aureus* (broth).	*Bact. coli* (broth).	pKa in water at 20°.	Per cent ionised pH 7·2.	Lipoid/water partition coefficient pH 7·2.
	(*a*)							
136	4-Aminopyridine	125	250	<2000	<2000	9·1	99	—
166	2-aminopyridine	<125	125	<500	<500	6·9	33	—
116	2-amino-4-phenylthiazole	250	500	<2000	<2000	(4·46)	0·18	—
118	2-*p*-aminophenyl pyridine	500	500	<2000	<2000	(5·17)	0·9	0·47
	(*b*)							
140	4-Aminoquinoline	500	500	<500	<500	9·1	99	0·02
48	4-amino-2-methylquinoline	1000	1000	1000	<1000	9·4	99·4	—
110	2-amino-5:7-dimethyl-1:8-naphthyridine	2000	2000	<2000	<2000	(7·07)	43	—
60	9-amino-5:6:7:8-tetrahydrophenanthridine	4000	4000	4000	2000	(7·80)	80	—
	(*c*)							
120	Acridine	4000	8000	<4000	<4000	(4·8)	0·4	—
36	5-aminoacridine	64000	64000	8000	8000	9·9	100	0·15
37	5-amino-1-methylacridine	128000	128000	8000	16000	10·2	100	0·19
111	4-amino-5:6-benzoquinoline	8000	8000	1000	2000	9·0	98·5	—
112	4-amino-6:7-benzoquinoline	16000	16000	4000	8000	9·7	100	—
113	4-amino-7:8-benzoquinoline	32000	32000	2000	8000	8·7	97	—
107	phenanthridine	4000	8000	<2000	<2000	4·3	0·12	—
58	9-aminophenanthridine	8000	16000	4000	4000	7·3	55·7	3·10
108	9-amino-1-methylphenanthridine	32000	64000	8000	4000	—	—	—
109	9-amino-3-methylphenanthridine	32000	64000	8000	4000	(7·44)	63·5	8·30
168	9-amino-1:3-dimethyl phenanthridine	16000	32000	<8000	<8000	(7·78)	79·0	47·0
61	9-amino-5:6:7:8-tetrahydro-3:4-benzphenanthridine	16000	16000	<4000	<4000	(7·0)	38·7	—
	(*d*)							
114	5-Amino-3:4-benzacridine	256000	256000	16000	8000	9·1	99	—
115	5-amino-1:2-benzacridine	256000	256000	32000	8000	8·8	97·5	4·90
131	5-amino-2:3-benzacridine	256000	256000	16000	8000	10·4	100	—
175	5-amino-9-methyl-1:2-benzacridine	128000	512000	<8000	<8000	(8·0)	88	—
183	5-amino-1-methyl-6:7-benzacridine	128000	256000	8000	<8000	(9·2)	99	—
	(*e*)							
178	5-Amino-1:2-8:9-dibenzacridine	32000	128000	16000	16000	(6·0)	6·0	—
184	5-amino-2:3-6:7-dibenzacridine	256000	512000	32000	<16000	(9·6)	99·6	—

TABLE II.—(a) *Nicotinamides ;* (b) *Nicotinamidines ;* (c) *Benziminazoles.*

Highest dilution expressed as 1/molarity of the substance completely inhibiting growth.

Lab. ref. Number.	Substance.	Structure.	Organisms and media. H37Rv serum-synthetic medium.	H37Rv synthetic medium.	*Staph. aureus* (broth).	*Bact. coli* (broth).	pKa in water at 20°.	Per cent ionised pH 7·2.
	(*a*) Nicotinamides	(XVI) ; R =						
161	Nicotinamide	H	<125	500	<500	<500	(3·55)	0·02
151	N-*p*-chlorophenylnicotinamide	C_6H_4Cl	2000	4000	<2000	<2000	—	—
154	N-*p*-phenoxyphenylnicotinamide	C_6H_4OPh	16000	32000	<8000	<8000	—	—
145	N-*p*-cyclohexylphenylnicotinamide	$C_6H_4C_6H_{11}$	<8000	<8000	,,	,,	—	—
147	N-4′-diphenylylnicotinamide	C_6H_4Ph	,,	,,	,,	,,	(2·89)	0·0
149	N-2′-dibenzofurylnicotinamide	$C_6H_3OC_6H_4$	,,	,,	,,	,,	—	—
	(*b*) Nicotinamidines	(XVII) ; R =						
162	Nicotinamidine	H	<125	125	<1000	<1000	(10·05)	99·8
150	N-*p*-chlorophenylnicotinamidine	C_6H_4Cl	<2000	<2000	<2000	<2000	—	—
152	N-*p*-ethoxyphenylnicotinamidine	C_6H_4OEt	,,	,,	,,	,,	—	—
153	N-*p*-phenoxyphenylnicotinamidine	C_6H_4OPh	4000	8000	,,	,,	—	—
144	N-*p*-cyclohexylphenylnicotinamidine	$C_6H_4C_6H_{11}$	8000	16000	<2000	<2000	—	—
146	N-4′-diphenylylnicotinamidine	C_6H_4Ph	4000	8000	,,	,,	(7·03)	28·5
148	N-2′-dibenzofurylnicotinamidine	$C_6H_3OC_6H_4$	<2000	<2000	,,	,,	—	—
	(*c*) Benziminazoles	(XVIII) ; R =						
35	2-morpholinomethylbenziminazole	$CH_2NC_4H_8O$	<2000	2000	<2000	<2000	—	—
88	2-phenoxymethylbenziminazole	CH_2OPh	2000	16000	,,	,,	—	—
103	2-benzyl benziminazole	CH_2Ph	4000	4000	,,	,,	—	—
87	2-cyclohex-1-enylbenziminazole	C_6H_9	4000	32000	<4000	<4000	—	—
54	2-phenylbenziminazole	Ph	<4000	8000	,,	,,	—	—
104	2-4′-diphenylylbenziminazole	C_6H_4Ph	<8000	<8000	<8000	<8000	(4·04)	0·07
63	2-1′-naphthylbenziminazole	$C_{10}H_7$	,,	8000	,,	,,	—	—

RESULTS.

Pyridines.—Table I*a* shows a series of amino-derivatives of pyridine (I), and the analogous 2-amino-4-phenylthiazole (No. 116 ; II). Activities are very low, ranging from M/125–M/500.

Quinolines.—Table I*b* deals with amines derived from quinoline (III), possessing two fused rings. It also includes a naphthyridine (No. 110 ; IV), and a tetrahydrophenanthridine (No. 60) in which the effective *flat* area of the parent molecule phenanthridine (V), has been reduced, by hydrogenation of one ring, to that available in quinoline (III). This series again shows only low activity, from M/500–M/4000.

Acridines and phenanthridines.—Table I*c* comprises amino compounds of acridine (VI), the isomeric benzoquinolines (VII, VIII and IX), and phenanthridine (V). The planar surface area has reached 40·8 sq. Å (Albert *et al.*, 1949 ; Cymerman-Craig *et al.*, 1954), and while the totally non-ionised parent compounds acridine (No. 120) and phenanthridine (No. 107) are inactive, the fully ionised amino compounds of this series show increased activity averaging M/16000–M/32000. The same activity is found in a tetrahydrobenzophenanthridine (No. 61 ; X) in which the molecular flat area has been reduced to that of benzoquinoline (VII).

Benzacridines.—A further marked enhancement is seen in Table I*d*, in which substances having 4 fused rings are listed. These highly ionised bases derived from the isomeric benzacridines (XI, XII and XIII) all exhibit very high activity, M/128000–M/256000.

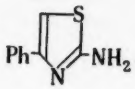
Dibenzacridines.—Introduction of a further fused benzene ring to give a dibenzacridine containing five fused aromatic rings was next examined. No amino-dibenzacridines were previously known, and two of these (No. 178 ; XIV and No. 184 ; XV) were prepared and examined. The former (No. 178) had low activity (M/32000) only ; it possessed pKa 6·0 and was thus only 6 per cent ionised at pH 7·2. The latter (No. 184) had pKa 9·6 and was fully ionised at the same pH ; it showed high activity (M/256000).

Nicotinamides, nicotinamidines and benziminazoles.—Table II deals with a series of nicotinamides (XVI) ; nicotinamidines (XVII) and benziminazoles (XVIII). Although the substituted compounds show activity greatly in excess of that of the parent compound of the series (nicotinamide), in only one case (No. 154) does activity reach M/16000. All compounds in Table II [with the exception of the highly water-soluble nicotinamidine (No. 162)] are non-ionised at pH 7·2 and were not further investigated. Chorine (1945) reports the antituberculous activity of nicotinamide as between M/60 and M/500, in good agreement with our results.

DISCUSSION.

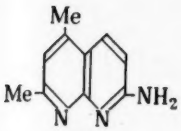
In Fig. 1, the tuberculostatic activities measured by minus log concentration (in presence of serum) of the amino-compounds in Table I containing 1, 2, 3, 4 and 5 fused rings are plotted against the flat surface area of the molecule in square Ångstrom units (1 Å $= 10^{-8}$ cm.). All results (with the exception of No. 184 and 178) fall near a straight line drawn through them, and this straight line has been expanded into a shaded area 0·3 unit above and below the mean values to allow for the twofold variation in the bacteriostatic end-point.

(I)



(II)

(III)



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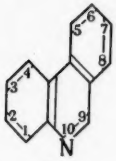

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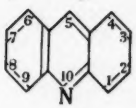

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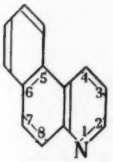

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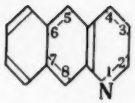
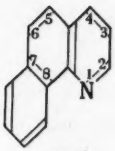


VIII



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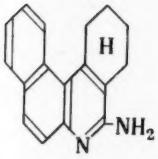

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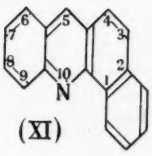

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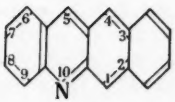
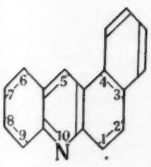


(XII)



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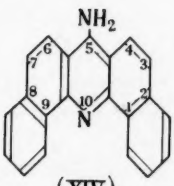

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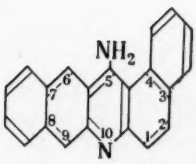

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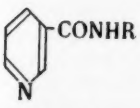

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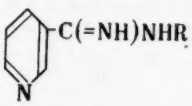

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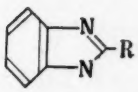

XVIII

No. 184 and 178 drop markedly below the shaded area of the graph, and it appears that as in the polycyclic primary amines (Cymerman-Craig *et al.*, 1954) a molecular size has been reached in these compounds where (due to limiting water-solubility) the maximum obtainable concentration in the "biophase" (in which biological action takes place) does not reach the minimum bacteriostatic concentration (Ferguson, 1939).

This explanation is confirmed by the fact that whereas in the non-ionised polycyclic primary amines (Part I) the maximum activity reached was M/32000 (3 rings) and the "Ferguson effect" then operated with 4-ring compounds, in the present work the maximum activity attained in the totally ionised heterocyclic

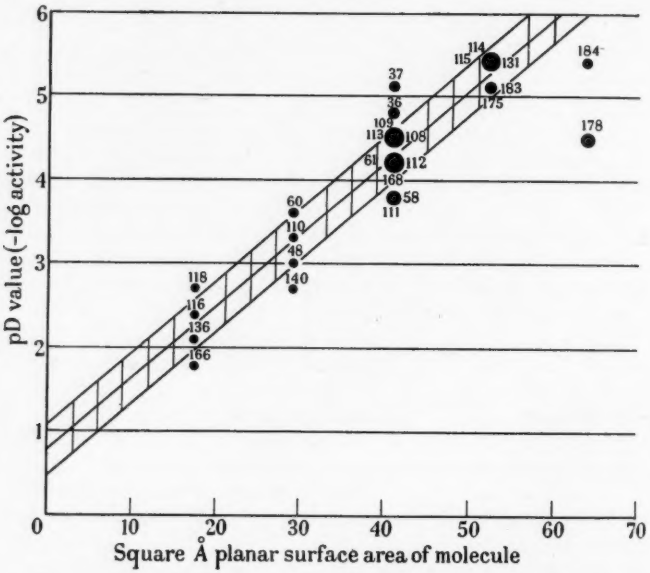

FIGURE.—The relationship of anti-tuberculous activity to molecular planar surface area for heterocyclic primary amines.

amines is the vastly greater value of M/256000 (4 rings). The cut-off in activity, characteristic of the "Ferguson effect" does not occur until 5-ring structures are reached.

The conclusion may now be reached that the anti-tuberculous action of aromatic and heterocyclic bases comprises (*a*) an adsorption factor, (*b*) interaction of a cationic group in the drug molecule with a negatively charged receptor site (or sites) in the organism, and (*c*) a phase-transfer of the drug molecule from an aqueous to a lipoid phase. When all three factors are satisfied anti-tuberculous action of the compounds becomes *specific*. Thus primary aromatic amines lack factor *b*, but possess factor *c*. As factor *a* increases with the number of rings, activity increases (Cymerman-Craig *et al.*, 1954) up to M/32000, but this is not specific for *Myco. tuberculosis*. This is shown by the comparable changes in

activity against other organisms and in the lowering of activity when the addition of serum provides an alternative surface for non-specific adsorption.

Similarly, in the diphenyl series dealt with in Part II, it was demonstrated that when the planar surface area (*i.e.*, factor *a*) was approximately constant, *both* factors *b* and *c* were prerequisites for optimum anti-tuberculous activity, and only then did the activity become specific for *Myco. tuberculosis*.

In the present series, factor *b* (pKa >8) is satisfied, factor *c* (partition coefficient not less than 0·2) is fulfilled when 3 rings are reached, and factor *a* (flat molecular surface area) increases with the number of rings. At the 3-ring structure level preferential adsorption becomes more specific for *Myco. tuberculosis* at some receptor site or sites where factors *b* and *c* then operate. This is shown by the lack of effect of serum on the activity of compounds in Table Ic, *d* and *e* (except in No. 178, where factor *b* is lacking).

The progressive inclusion of the 3 factors discussed clearly results in a progressive increase in the anti-tuberculous activity in this series from M/125 to M/256000, *i.e.*, a 2000-fold range.

SUMMARY.

Forty-seven aromatic heterocyclic bases have been examined for their anti-tuberculous activity.

Twenty-seven of these compounds, possessing a primary amino group, have been investigated in detail and their anti-tuberculous activity correlated with three physico-chemical properties.

In agreement with previous results, anti-tuberculous activity has been shown to depend on a high degree of basic strength, and to be proportional to the planar surface area of the molecule.

The overall increase in anti-tuberculous activity achieved by manipulation of these physico-chemical factors is of the order of 2000-fold, and the fulfilment of these criteria introduces marked specificity of anti-tuberculous action.

The authors are indebted to the National Health and Medical Research Council and the Rockefeller Foundation for financial support, and also wish to thank Miss M. Pinkerton for assistance in testing some of the substances.

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THE MECHANISM OF ARTHUS REACTIONS. I. THE RÔLE OF POLYMORPHONUCLEAR LEUCOCYTES AND OTHER FACTORS IN REVERSED PASSIVE ARTHUS REACTIONS IN RABBITS.

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THE Arthus reaction, one of the classical types of allergic reaction, is evoked by the introduction of antigen into the tissues (usually into the skin) of animals whose plasma contains precipitating antibodies. The experimental animals have usually been rabbits, because rabbits are excellent producers of precipitating antibodies, but characteristic reactions occur in other species, including man, dog, guinea-pig and rat. It has been abundantly shown that ability to produce an Arthus reaction can be passively transferred by injection of either homologous or heterologous precipitating antibodies, and there is good evidence that neither non-precipitating antibodies nor cell-borne antibodies (Culbertson, 1935 ; Cannon and Marshall, 1941 ; Fischel and Kabat, 1947) are essential, or are even involved in the reaction. The macroscopic appearance of an Arthus-type reaction depends on its severity, but there is characteristically an inflammatory reaction with erythema, oedema and a varying degree of haemorrhage and necrosis, apparent after 1–2 hr. and maximum after 8–24 hr. or more, after which it slowly subsides. In the early stages there is massive oedema and invasion of the tissues at the injection site with polymorphonuclear leucocytes (polymorphs), which are concentrated mainly in and around the small blood vessels but also in the connective tissue and other structures. There are many thrombi consisting of a mixture of platelets and polymorphs, and the vessel walls (particularly those of the venules) are necrotic, with varying degrees of haemorrhage. Mononuclear cells are not prominent during the early stages. After 12–24 hr. the number of polymorphs declines rapidly, and the polymorph invasion gives way to infiltration by mononuclear cells, which, according to Gell and Hinde (1954), arise partly by invasion from the blood stream and partly by proliferation of local histiocytes.

Thus the Arthus reaction, as normally elicited, contains at least two elements, namely the consequences of the local combination of antigen with antibody, and of the local introduction of a foreign protein. It was the aim of this work to study the first element only. By using reversed passive Arthus (R.P.A.) reactions the problem can be much simplified. In this variation of the reaction, antigen is given intravenously into normal animals, and antibody is injected soon afterwards into the skin (see Fischel and Kabat, 1947). There is no question of any pre-existing cellular hypersensitivity in the test animal, and, if homologous antibody is used for injection into the skin, there is no local foreign protein reaction at the injection site, since the antigen is distributed generally throughout the circulation. Furthermore (Humphrey, 1951) R.P.A. reactions can be repeatedly reproduced in different skin sites on the same animal for periods up to six days, and it is possible

to assess the effects of various treatments against control periods in the same animals. There is the added advantage that variations in antibody levels, which complicate experiments using active immunisation, are eliminated.

R.P.A. reactions in the rabbit are uninfluenced by antihistamine drugs, though they are somewhat diminished by large doses of sodium salicylate (Smith and Humphrey, 1949) and considerably diminished by single doses of cortisone or ACTH (Humphrey, 1951). These observations, together with the time relationships of the reactions and the observed histological changes, suggested that the cellular invasion might be an essential part of the reaction, and not merely a consequence of secondary tissue damage. A means of investigating this point was suggested by Thomas and Stetson's (1949) use of nitrogen mustard (HN2) temporarily to diminish circulating polymorphs, during their studies of the Shwartzman reaction in the rabbit. The effect of suitable doses of HN2 was striking—R.P.A. reactions in rabbits being virtually abolished so long as the polymorph count was low.

While this work was in progress, Stetson (1951) extended his study of the Shwartzman reaction to include Arthus reactions. He drew attention to the similarity of the two reactions, both in their behaviour towards HN2 treatment and in their early histological appearances and suggested that in both instances an abnormal metabolic process developed in the injected skin areas which increased the vulnerability of the local capillaries and venules to leucocyte-platelet thrombi. The circumstantial evidence that invasion by polymorphs is an important link in the chain of events which make up the Arthus reaction is quite good. Before coming to definite conclusions, however, it seemed wise to use other agents than HN2 for depleting the animals of polymorphs, and to investigate other species besides rabbits. It was also desirable to show that replacement of the polymorphs in a depleted animal would restore the capacity to produce a reaction, since such a demonstration would prove that they were essential.

The following papers describe a study of R.P.A. reactions in the rabbit and the guinea-pig, and of the effect upon them of agents which affected the numbers or the properties of the circulating polymorphs and platelets. Although attempts to replace polymorphs by transfusion to deficient animals failed, the remaining evidence suggests that polymorphs are essential for oedema formation and for vascular damage in the rabbit. The reaction in the guinea-pig, which is more complicated and in only part of which are polymorphs concerned, is described in a separate paper.

METHODS.

Animals.

Mature rabbits of either sex, from the Sandylop and albino strains maintained at the National Institute for Medical Research, London, were used throughout. They were fed on pelleted diet No. 81 of Bruce and Parkes (1946) supplemented with hay.

Antigens and antibodies.

Antigens used were crystallised bovine serum albumin (Armour), 5 × recrystallised hen ovalbumin, human γ-globulin and a preparation of the capsular polysaccharide of pneumococcus Type III. Antisera were prepared by immunisation of rabbits with the antigens, either incorporated in Freund's adjuvant mixture, or adsorbed on alum, or, in the case of anti-pneumococcus sera, by intravenous injection of formalin-killed whole organisms. The sera contained 4·5–15 mg. of specifically precipitable antibody per ml.

Technique of reversed passive Arthus reactions.

Normal rabbits, whose bellies had previously been depilated, were injected, intravenously with 20–30 mg. of the protein antigens, or 2–3 mg. pneumococcus polysaccharide. After ½–1 hr., 0·5 ml. of antiserum, diluted to contain 3–5 mg. antibody/ml., was injected intracutaneously on each side of the belly. The rabbits were examined at intervals, and the oedema volumes measured as described by Humphrey (1951). Four days later, except where otherwise stated, a further injection of antigen was given and the procedure was repeated at different, but corresponding sites on the belly wall.

When leakage of Pontamine Blue was to be studied, the rabbits were given 5 per cent Pontamine Sky Blue 6BX intravenously in a dose of 0·6 ml./kg. This was usually injected ¼ hr. before the test antibodies, but in certain experiments injection was delayed until after the Arthus reactions had begun to develop.

Collection of polymorphonuclear leucocyte exudates.

Exudates were induced in normal rabbits by intraperitoneal injection of 150 ml. 3 per cent peptone in 0·9 per cent NaCl, previously warmed to 37°. After 12 hr. a further 50 ml. was injected, and the exudates were aspirated 3 hr. later, using the technique described by Mackaness (1952). All glassware used was treated with silicone, but a variable amount of fibrin clot generally formed during the subsequent manipulations. When the cells were to be used for transfusion within an hour of collection, clot formation was prevented by addition of sufficient sodium citrate or sodium ethylenediamine tetra-acetate, and the exudate was cooled slowly by standing in a refrigerator. The cells were deposited by centrifugation at 800 *g.* and, after removal of most of the supernatant fluid, were re-suspended in approximately 10 ml. Differential counts showed that 85–95 per cent were neutrophil polymorphs. Nearly all were actively motile when examined on a warm stage microscope.

When it was desired to transfuse exudate cells produced in a particular animal back into the same animal after depletion of its circulating polymorphs by nitrogen mustard, it was necessary to maintain the cells viable for at least 8 days—allowing 4 days for the animal to recover from the peritoneal lavage and for the scar to unite, and 4 days for the HN2 to take effect. With the help of Dr. Audrey Smith, of this Institute, it was found that exudate cells would remain largely viable—as judged by ability to show amoeboid movements and to ingest staphylococci—if they were stored as a deposit in the original exudate fluid at the bottom of a centrifuge tube at 2°.

Fewer cells remained viable when anticoagulants were added, but without them there occurred extensive formation of fibrin threads and clumping of the leucocytes on the threads. In order to obtain suspensions of dispersed cells suitable for intravenous injection, the following procedure was adopted. The exudate was aspirated into sterile flasks, and the peritoneal cavity washed out with Ringer solution containing 0·1 per cent gelatin and 10 u./ml. penicillin. The combined exudate and washings were agitated gently at room temperature while a fine clot formed, and they were then poured through silicone-treated muslin which retained most of the fibrin. After cooling slowly to 2°, the cells were deposited by centrifuging at 800 *g.* and were stored at 2° for 8–14 days. After storage for this time, 70–80 per cent appeared to be fully viable, and about 5–10 per cent were dead, when examined by phase contrast illumination on a warm stage. The cells were in clumps, however, and although they could be partly dispersed by gentle agitation for ½ hr. at 37°, it was found more convenient to treat the suspension with 2000 units streptokinase + 500 units streptodornase (Burroughs Wellcome and Co.) for 5 min. at 37° immediately before intravenous injection into the rabbits. This treatment yielded a uniform suspension which caused no apparent ill effects on the recipients, although—as is described below—no rise in the systemic polymorph counts resulted.

Dosage of nitrogen mustard.

" Mustine " (Boots Pure Drug Co.) was injected intravenously immediately after solution, over a dose range 0·5–2·0 mg./kg. and polymorph counts were made daily for 6 days. The changes in white cell counts followed a constant pattern, which is reported in the text of the paper below. Although 1 mg./kg. was usually sufficient to lower the polymorph count below 50/c.mm. on the fourth day, 1·75 mg./kg. was chosen as the dose which caused a consistent fall without any obvious general ill effects on the animals.

RESULTS.

Experiments using Nitrogen Mustard.

Oedema in R.P.A. reactions.

The typical course of development of oedema at a reaction site is shown in Fig. 1. The volume of measurable oedema increases rapidly, reaching a maximum at 7–10 hr. This pattern is constant, especially when duplicate sites are injected, and repeatable so long as the amount and volume of antibody are standard. In all experiments oedema volumes were measured at regular intervals, but the 7 hr.

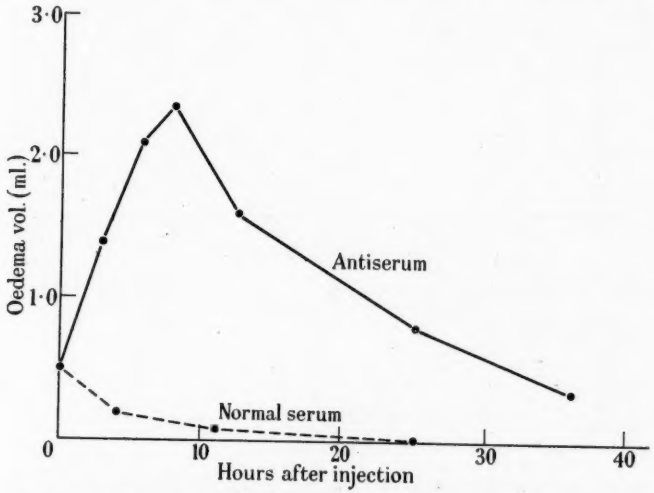


FIG. 1.—Time course of oedema at site of reversed passive Arthus reaction.

reading was arbitrarily chosen as the standard for convenience. Fig. 2 and 3 summarise the results of many experiments carried out with HN2, and show the relationship between the oedema at 7 hr. and the numbers of circulating neutrophil polymorphs and of mononuclear cells (lymphocytes + monocytes) respectively. Each point is the mean of two reaction sites, and every animal tested is represented twice—once during the test and once during the control period. It is evident that there is no correlation between oedema and monocuclear cells, but a definite correlation with polymorphs.

When the polymorph count approached zero, the visible reaction was abolished; it increased with increasing numbers of circulating polymorphs until the count was about 1500/c.mm. Above this level the response, though variable, was probably maximal. (The variability shown is not solely due to variation from animal to animal, but is enhanced by the inclusion in these scatter diagrams of experiments using several different antigen-antibody systems and with somewhat varying doses of antibody.)

After an injection of HN2 (1·5–1·75 mg./kg.) the number of circulating polymorphs does not drop greatly during the first two days, but falls precipitately

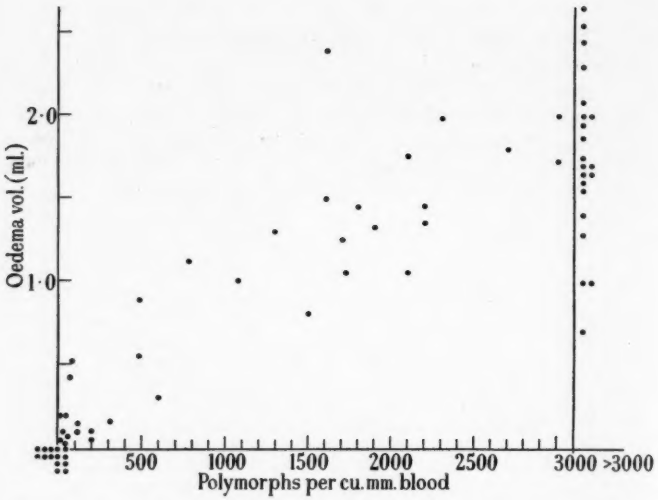


FIG. 2.—Correlation between oedema volume after 7 hr. at site of R.P.A. reaction and blood neutrophil count.

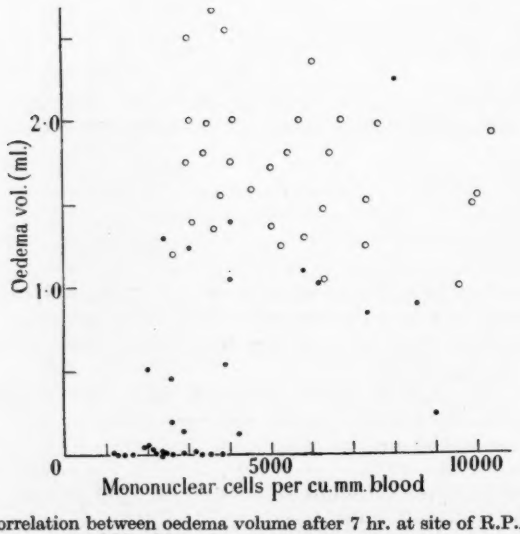


FIG. 3.—Correlation between oedema volume after 7 hr. at site of R.P.A. reaction and blood lymphocyte + monocyte count.

● 4 days after HN2.
○ 8 „ „ „

thereafter. By the 5th to the 6th day regeneration is occurring, and the blood contains many primitive as well as normal cells. After 8 days, the blood picture is restored to approximately normal. This sequence of events is probably brought about by an immediate toxic effect of the HN2, which prevents any new production of granulocytes by the bone marrow for about 5 days, although it does not impair the function of mature cells, which continue in the circulation and disappear with a normal half life. Repeated duplicate tests were made in two rabbits at intervals after HN2 administration, and Fig. 4 shows that the size of the reaction closely

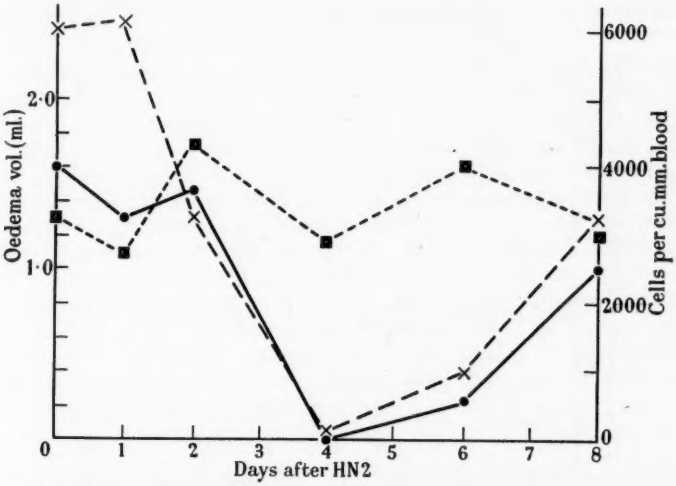


FIG. 4.—Correlation between blood white cell count and oedema volume after 7 hr. at site of R.P.A. reaction performed in same rabbit at varying times after HN2.

● oedema volume.
× neutrophil polymorph count.
■ mononuclear cell count.

followed the polymorph count. Thus the effect of HN2 on the reaction shows the same latency, and disappears at the same rate as its effect on polymorphs.

The correlation in time between the number of circulating polymorphs and ability to elicit an Arthus reaction was well demonstrated in another experiment in which the direct passive Arthus reaction was being studied. Two rabbits which had received HN2 4 days previously were injected intravenously with 150 mg. homologous antibodies against Type III pneumococcus polysaccharide. Intracutaneous injections of 0·5 mg. antigen 2 hr. later produced no reaction, although in control rabbits, immunised in the same way, vigorous haemorrhagic Arthus reactions appeared. Two days later, however, when polymorphs began to return to the circulation in the treated animals, the injected sites showed typical moderately severe reactions. Presumably the reaction of antigen with antibody had occurred in treated and controls alike, but any changes produced were harmless

until polymorphs arrived on the scene. Similar observations were made by Stetson (1951) in actively immunised rabbits.

Control experiments with HN2.

Nitrogen mustard, in doses sufficient to eliminate neutrophil polymorphs from the circulation, is likely to affect other tissues besides the bone marrow—*e.g.*, liver, spleen, adrenals, gastro-intestinal tract, as well as the skin in which the reactions are elicited. As was shown by Becker (1948) and by Stetson and Good (1951) in their studies of the Shwartzman phenomenon, the neutrophil depletion after intravenous HN2 administration can to a large extent be prevented if the

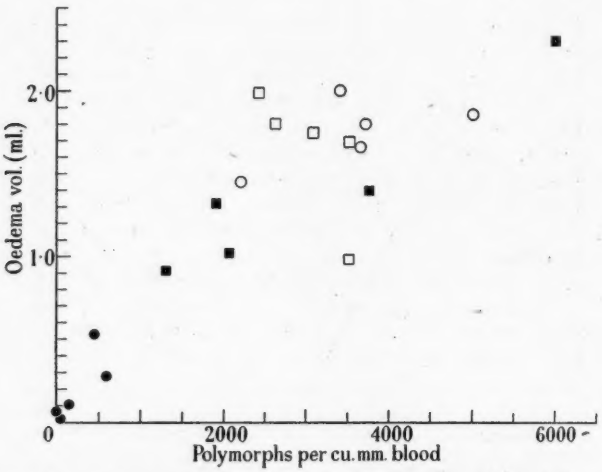


FIG. 5.—Correlation between oedema volume after 7 hr. at site of R.P.A. reaction and blood neutrophil count in rabbits treated with HN2 with and without clamping of the aorta.

○ Aorta not clamped 8 days after HN2.
● „ „ „ 4 „ „ „
□ „ clamped 8 days after HN2.
■ „ „ 4 „ „ „

abdominal aorta of the rabbit is clamped just above the bifurcation for 5 min. during and after the injection of HN2. By this means the legs, including their femoral bone marrow, escape the toxic effects of HN2, although the rest of the body is subjected to the drug. Animals which were injected while their aorta was clamped showed a reduction in neutrophil leucocyte count to about half the on groups of 5 animals subjected to the following treatments : HN2 injection with laparotomy, but without clamping the aorta ; HN2 injection with clamping of the pre-operation levels. R.P.A. reactions were performed 4 and 8 days after injection aorta ; saline injection with clamping of the aorta. This last group showed no difference from unoperated normal controls, and are not included. The results obtained in the first two groups are shown in Fig. 5.

It is evident that, so far as ability to elicit an Arthus reaction is concerned, HN2 had no effect when the aorta was clamped, even though the skin sites to be tested had been subjected to its influence. Furthermore, histological examination of biopsies of the reaction sites revealed no difference between the reactions in control animals and in those which had received HN2 with the aorta clamped, although the reaction was virtually abolished in the animals which received HN2 under laparotomy only (Fig. 6). These observations provide strong evidence, although not complete proof, that HN2 abolishes the reaction solely by virtue of its effect on the polymorphs.

Effect of HN2 on platelets and on complement.

In view of the facts that leucocyte-platelet thrombi are a prominent feature of Arthus reactions, and that rabbit platelets contain large amounts of histamine and 5-hydroxytryptamine releasable by antigen-antibody reactions (Humphrey and Jaques, 1953), it was necessary to investigate the effect of HN2 on platelets as well as on polymorphonuclear cells. In the doses used the numbers of platelets were reduced from about 700,000/c.mm. to between about 250,000 and 500,000/c.mm. Their histamine content, estimated biologically on washed buffy layer of the blood, was always within normal range. It seems improbable that the small reduction in numbers could account for the observed changes.

Since complement fixation is a feature of antigen-antibody reactions in the rabbit (Stavitsky, Stavitsky and Ecker, 1949) the effect of HN2 administration on the level of haemolytic complement was examined. In four animals, which showed an almost complete absence of polymorphs 4 days after HN2, no significant changes in blood complement were found.

Effect of HN2 on reactions to histamine, leukotaxine and the histamine liberator 48–80.

It was possible that the effective action of HN2 might be not so much upon the polymorphs as upon the reactivity of the small blood vessels to some unknown product of antigen-antibody reactions. Miles and Miles (1952) showed that the susceptibility of the cutaneous vessels of guinea-pigs to various agents could be studied by measurement of the area of blueing produced by injections of graded quantities of these agents, under standardised conditions in the skin of animals which had previously received intravenous injections of Pontamine Blue. The substances studied by Miles and Miles, namely, histamine, leukotaxine and the histamine liberator 48–80, were chosen as representative capillary-damaging agents.

In the present experiments they were injected in graded doses over the range 0·05–12, 0·8–200, and 0·05–50 μg. respectively, distributed at random in the skins of 10 rabbits, 5 of which had received HN2 previously and 5 were controls. All the rabbits had received intravenous injections of 0·6 ml./kg. 5 per cent Pontamine Sky Blue 6BX $\frac{1}{2}$ hr. beforehand. Measurements of the area and intensity of blueing at the injected skin sites revealed considerable differences between animals of the same group, but no significant difference between the groups.

The results make it very improbable that changes in resistance to capillary damaging agents could explain the effects of HN2 on Arthus reactions.

Attempted Replacement of Polymorphs in Depleted Animals.

Systemic injections.

Numerous attempts were made to raise the number of circulating polymorphs in depleted animals by transfusion of washed or unwashed cells obtained from peritoneal exudates (see Methods). In 6 cases the exudates had been obtained from the recipient animals themselves before HN2 administration and had been preserved for 8–14 days at 2°, while in 4 the exudates were freshly obtained from normal rabbits of the same breed. Although the transfused cells were in good physiological condition (judged by motility, phagocytic activity, and failure to stain with methylene blue), and were not clumped, and although the number of cells injected was sufficient to raise the polymorph counts in the recipient animals to or above normal levels, in only one instance was the level raised significantly.

No difference was observed between the results of injection into the marginal vein of the ear or of injection into the left side of the heart. Presumably the cells from the peritoneal exudates were in some way altered so as to adhere to the walls of the blood vessels (see Heinle, Weisberger and Guyton, 1950). In the single animal which showed a rise after transfusion, intravenous injection of 2×10^9 fresh cells from another donor produced a sustained increase in the polymorph count from 20 to 480/c.mm. The mean volume of oedema after 7 hr. at the R.P.A. sites in this animal was 0·54 ml. compared with 2·0 ml. when the reaction was repeated 4 days later at a time when the polymorph count was 3400/c.mm. A volume of 0·5 ml. of oedema is about the amount expected at a polymorph level of 400/c.mm. rather than at a level of 20 c.mm., but a single partly successful experiment cannot be claimed as proof that polymorphs are essential for development of the reaction.

Local injection.

Although local injection of polymorphs mixed with the test sera does not achieve the same distribution of these cells as results from invasion from the bloodstream, some experiments were made to test their effect in restoring reversed passive Arthus reactions in depleted animals. When 10^8 fresh exudate cells (*i.e.*, the amount present in about 20 ml. blood) were added to antiserum or to normal serum and injected intracutaneously into depleted animals which had already received antigen, some increase in oedema was observed at 6–7 hr. Table I shows the effect in 3 rabbits of added polymorphs, the oedema volumes being expressed as a percentage of the full-size reactions obtained on re-testing the rabbits 4 days later when polymorphs had returned to the circulation. Addition

EXPLANATION OF PLATES.

FIG. 6–11 show site of R.P.A. reaction.

FIG. 6.—Polymorph-depleted rabbit : 5 hr. (× 15).

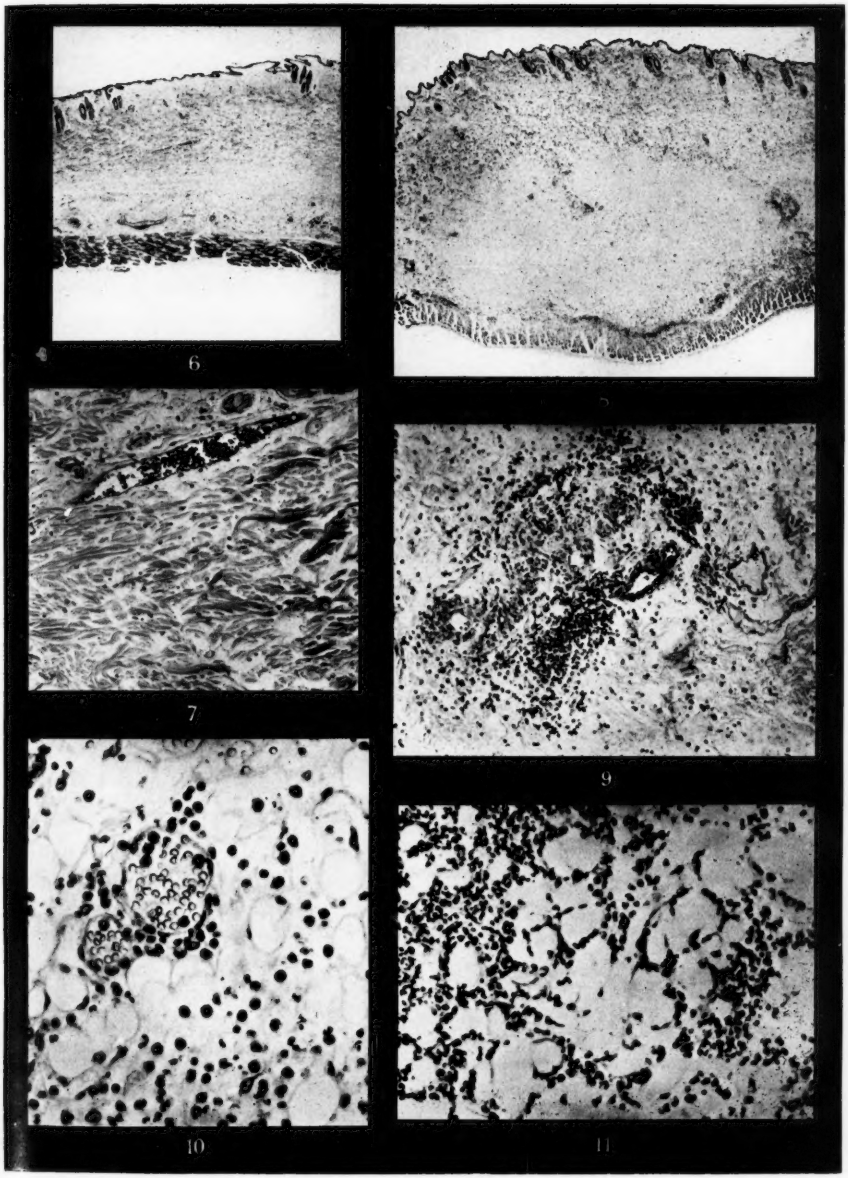
FIG. 7.—High power of Fig. 6 showing absence of perivascular infiltration (× 130).

FIG. 8.—Undepleted rabbit 6 hr. (× 15).

FIG. 9.—High power of Fig. 8 showing polymorph infiltration and early vascular necrosis (× 130).

FIG. 10.—Polymorph-depleted rabbit ; 24 hr.; large dose of antibodies. Shows pure mononuclear cell infiltration (× 130).

FIG. 11.—Undepleted rabbit 24 hr. ; large dose of antibodies. Shows mixed mononuclear cell infiltration and pyknotic polymorphs (× 130).



Humphrey.

of polymorphs at the site of injection partly, but never completely, restored the reaction.

TABLE I.—*Mean Oedema Volumes after 6–7 hr. at Sites of R.P.A. Reactions in 3 Depleted Rabbits, and 2 Controls Expressed as Percentage of Mean Volume of Repeat Reactions 4 Days Later.*

	Depleted.	Controls.
Antiserum alone	4	81
Normal serum alone	1	—
10^8 polymorphs in saline	6	8
Normal serum and 10^8 polymorphs	24	—
Antiserum and 10^8 polymorphs	42	96

The mean polymorph count in the depleted rabbits was 26 per c.mm. The mean oedema volume in repeat reactions was 2·2 ml.

Capillary Permeability Changes at R.P.A. Sites.

The changes in capillary permeability at the sites of R.P.A. reactions can be studied in rabbits which have been given intravenous injections of Pontamine Blue (30 mg./kg.) ½ hr. beforehand. The sequence of events was fairly constant. For the first ½ hr. little or no blueing was observed at the reaction sites, but by 1 hr. a definite blue area appeared at the centre, and this area became more deeply stained during the next 3–4 hr. The peripheral area around the site meanwhile also became stained blue, although never so intensely as the central area. After 5 hr. no further changes occurred and the appearance (with the doses used) resembled a target, with a dark blue centre 10–15 mm. across, and a paler blue periphery 20–25 mm. in diameter. The area of visible oedema extended beyond the blue area. In the absence of antigen most whole sera caused little or no blueing, although normal or antibody-rich gamma globulin fractions (prepared by precipitation with Na_2SO_4) always caused deep central blueing, even when no antigen was administered and no oedema developed. In rabbits treated with HN2, the blueing at reaction sites due to the specific antigen-antibody reactions was almost completely abolished—*i.e.*, it was reduced almost to that produced by injection of the serum alone into normal animals. This degree of reduction was greater than that previously observed after single doses of cortisone (Humphrey, 1951).

Histological Changes.

The histological changes are described only in so far as they shed light on the pathogenesis of the reaction. Plates I–IV Fig. 6–9 show the striking difference after 7 hr. between the almost normal appearance of the skin reaction sites in depleted animals and the intense inflammatory changes in the untreated controls. The changes in the latter are essentially similar to those described in the review by Gell and Hinde (1954), except that the mononuclear cell reaction was quite inconspicuous up to 8 hr. Such small perivascular mononuclear cell collections as did occur were observed equally in both treated and control groups.

When reaction sites were examined after 24 and 48 hr., however, there was much less to distinguish those in polymorph-depleted animals from undepleted controls, particularly when large amounts of antiserum were used. Control reaction sites at 24 hr. were still grossly oedematous, and contained many neutrophil polymorphs, most of which showed pyknotic degeneration ; after 48 hr. the

neutrophil polymorphs were all pyknotic, although intact eosinophils were fairly numerous, and the oedema was much less. In addition to these features, which were confined to the control animals, there was present in animals of both series a considerable mononuclear cell proliferation and invasion, similar to that described by Gell and Hinde at sites of direct Arthus reactions. This was diffuse throughout the dermis and the underlying *panniculus carnosus*, but particularly marked around small blood vessels (Fig. 10 and 11). By 48 hr. thrombosis of the venules, which was still evident at 24 hr., was relatively rare.

It appears, therefore, that the events at sites of reversed passive Arthus reactions take broadly the same course as at those of direct Arthus reactions although perhaps more slowly. Furthermore, suppression of the neutrophil polymorph invasion, though it greatly diminished thrombosis, oedema and vascular necrosis, did not prevent the succeeding phase of the reaction from following its usual course. The phase of mononuclear cell proliferation and invasion must therefore be directly related to the antigen-antibody reaction, and not secondary to the neutrophil polymorph phase.

The Effect of Other Treatments on R.P.A. Reactions.

Bacterial polysaccharide.

A potent pyrogenic polysaccharide, prepared from *Chromobacterium prodigiosum* by a modification of the method of Shear and Turner (1943), when injected intravenously in doses of 15 μg/kg., caused a fall in the polymorph counts to between 100 and 500/c.mm. and in lymphocyte counts to 1200–2100/c.mm. This fall occurred within $\frac{1}{2}$ hr. and lasted 4–5 hr., during which period neither oedema nor significant histological changes occurred at the sites of R.P.A. reactions. Interpretation of these results was complicated, however, by a prolonged fall in peripheral blood pressure, which accompanied the leucopenia and persisted for at least 12 hr.

Heparin.

Since Good and Thomas (1952) and Cluff and Berthrong (1953) claim that administration of heparin in large doses prevents local Shwartzman reactions in rabbits, it seemed worth while to test whether R.P.A. reactions would also be suppressed. Even very large amounts of heparin (British Drug Houses, Ltd.), however, reduced the oedema only by about one half, and did not alter their general appearance. The results of two cross-over experiments are given in Table II.

TABLE II.—*Effect of Heparin on Development of Oedema at Sites of R.P.A. Reactions.*

Total amount of heparin.	Dosage (I.V.).	Volume of oedema (ml.) at 6 hr.	
		Control period.	Heparin period
1,600 i.u.	2 × 800 i.u. ½ hourly	2·0	2·2
4,800 „	6 × 800 „ hourly	1·6	1·9
48,000 „	6 × 8000 „ „	2·25	1·45
48,000 „	6 × 8000 „ „	2·9	1·1

The rabbits were tested later with a different antigen-antibody system, 4 days after HN2 administration, and gave negligible reactions.

Denervation.

Since capillary tone, and to some extent permeability, are under nervous control, the effect of denervation on R.P.A. reactions was studied. The ears of three Sandylop rabbits were denervated on one side by removal of the stellate and superior cervical sympathetic ganglia, with and without section of the second cervical sensory nerve. R.P.A. reactions were induced in both ears by the usual technique and the results are shown in Table III. Although oedema was more rapid and severe on the denervated than on the control sides, the reactions appeared similar, and the more explosive development in the operated ears was probably due to the increased blood flow through them.

TABLE III.—*Development of Oedema at Sites of R.P.A. Reactions in the Ears of Rabbits which had been Denervated on One Side.*

Time.	Mean oedema vol. (ml.)	
	Unoperated side.	Denervated side.
4 hr. . .	1·0	1·7
6 hr. . .	1·4	2·7
23 hr. . .	1·4	0·6
32 hr. . .	1·2	0·2

DISCUSSION.

Large molecules, such as serum albumin, introduced directly into the circulation take 10–20 hours to come into complete equilibrium with the lymph protein in the tissue spaces (Wasserman and Mayerson, 1951). In the experiments described above, antigen was usually injected in amounts 200–300 times greater than those required to combine with the antibody, but at the actual site of injection antibody would certainly be initially present in excess. Thus there would be a pool of antibody, tending to diffuse slowly into the venous capillaries and lymphatics, meeting antigen diffusing steadily from the capillaries into the tissue spaces. Although we do not know the detailed mechanism by which proteins pass from plasma to lymph (see Pappenheimer, 1953) there is no doubt that the meeting place of antigen with antibody must be in or around the small blood vessels. The sequence of events following introduction of horse serum into sensitised rabbits, either locally into the tissues or into the systemic circulation, was observed by Abell and Schenck (1938) and by Ebert and Wissler (1951) using the ear chamber technique, Some 15–20 minutes after introduction of antigen into the moat of the chamber they observed intermittent and asynchronous contraction of arterioles, with complete obliteration of the lumina, which resulted in intermittent circulation of blood through the area. After 1 hour clumps of leucocytes began to appear, and this was rapidly followed by adherence of leucocytes to the walls of peripheral capillaries and venules. Both intermittent contraction of peripheral arterioles and increasing adherence of leucocytes to the walls of all the vessels continued for 2½ hours. About this time there began an emigration of leucocytes into the tissues, which continued until next day, by which time clumping in the vessels had ceased. When antigen was introduced repeatedly, at weekly intervals, the process became more marked during the later introductions, and intense leucocytic emigration was accompanied by extravasation of erythrocytes, and by injury to the endothelium and actual destruction of the vascular wall.

The experiments reported above, in conjunction with Abell and Schenck's direct observations, suggest that the sequence of events is as follows.

Stage I.—A slowly developing, but relatively minor, damage to the local blood vessels, resulting from the union of antigen with antibody at the site and causing stickiness of the endothelium and arteriolar spasm. The mechanism of this damage is at present unknown, although it may perhaps be related to the changes occurring in immune plasma on addition of antigen, which cause release of 5-hydroxytryptamine and histamine from platelets (Humphrey and Jaques, 1953).

An increase in capillary permeability appears when the endothelial stickiness begins, but in the absence of Stage II it is transient and of limited extent. It is possible that the effect of cortisone administration (Humphrey, 1951) is to diminish the endothelial changes, and thereby to reduce the sequelae of the next stage.

Stage II.—Adherence of polymorphonuclear leucocytes to the walls, and intravascular clumping of leucocytes and platelets. Histamine and 5-hydroxytryptamine are probably released in significant quantities from the platelets, causing further increase in arteriolar spasm and in capillary permeability.

In the presence of sufficient antigen and antibody this process continues, and as the polymorphs accumulate the vessels become more or less plugged with cells and destruction of vessel walls occurs. This is particularly marked in venules and capillaries. At this stage there is extensive emigration of polymorphs into the surrounding tissues, and gross oedema becomes apparent.

Experiments not reported here indicated that oedema formation and emigration of polymorphs continue after leakage of Pontamine Blue from patent capillaries has ceased, the badly damaged vessels having meanwhile become completely plugged so that gross leakage does not occur fom them. The fluid which continues to accumulate in the neighbouring tissues (whose lymphatic channels are distended) presumably contains little plasma protein. Its accumulation implies that lymph drainage is obstructed, although the cause of this was not ascertained. Partial blocking by polymorphs is a possible cause.

All the changes of this stage were abolished or very greatly reduced by treatment with HN2 when, but only when, the circulating polymorph count was drastically lowered. This indicates that polymorph accumulation is essential for the vascular destruction and oedema, which are the main features of severe reactions. Adherence of platelets to the vascular endothelium could be seen in treated animals but was not extensive, and it is suggested that platelets are not essential for the destructive phase of the reaction, although their contained 5-hydroxytryptamine and histamine may well play a part by causing spasm and endothelial stickiness. Stage II is at its height 6–12 hours after the beginning of the reaction (depending on the severity) and it gradually gives place to Stage III.

Stage III.—This stage is characterised by invasion of the reaction site by macrophages, and by local proliferation of histiocytes. It is accompanied by degeneration and disappearance of the neutrophil polymorphs, by removal of leucocyte-platelet thrombi, and by repair of the vascular damage of Stage II by proliferation of endothelial cells. Since no histological observations were made beyond 48 hours, it is not possible to state whether the mononuclear cell collections develop into nests of plasma cells, as described by Gell and Hinde in direct Arthus reactions. Although the biochemical events which lead up to Stage III are unknown, it is interesting that the polymorph invasion of Stage II does not appear to be an essential factor.

The experimental work described above has largely been concerned with the rôle of neutrophil polymorphs, but it leaves the mechanism unexplained. Perhaps the most striking thing is the enormous number of these cells which are present at the reaction site. Of their known activities or properties any of the following might be important: proteolytic and other hydrolysing enzymes (Weiss, Kaplan and Larson, 1938; Barnes, 1940); very active anaerobic glycolysis with lactic acid production (Stetson, 1951); intense reducing activity (Rouser, 1951). Their concentration could well be sufficient locally to overwhelm any homoeostatic mechanism, and to cause a profound change in environmental conditions of the tissue cells.

SUMMARY.

A quantitative study was made of reversed passive Arthus reactions in rabbits, whose circulating neutrophil polymorphonuclear leucocytes had been reduced to very low levels by administration of nitrogen mustard.

In the absence of neutrophils, oedema formation was completely suppressed, and the early increase in local vascular permeability was much diminished.

Histological examination showed that, in the absence of circulating neutrophils, there was no local neutrophil infiltration of the reaction site, and very much less vascular necrosis. Mononuclear cell infiltration in the later stages was not affected compared with normal controls.

In rabbits which received nitrogen mustard, but whose neutrophil count was maintained by protection of the femoral bone marrow, Arthus reactions occurred normally.

The suppression of oedema did not depend on changes in other formed elements of the blood, or in haemolytic complement, or in skin reactivity to histamine 48–80 or leukotaxine.

Attempts to restore the level of circulating neutrophils in depleted rabbits, by transfusion of suspensions of fresh homologous cells or of stored autogenous cells, were largely unsuccessful.

The effect of other factors on the development of reversed passive Arthus reactions was studied, in an attempt to elucidate their mechanism. Heparin in very large doses caused only slight reduction in intensity. A bacterial polysaccharide which produced neutropenia very greatly reduced the reactions, but the observations were complicated by a prolonged fall in blood pressure. Arthus reactions in completely denervated ears differed from those in normal ears only by a more rapid course.

It is suggested that the reaction comprises three stages:

(I) Relatively rapid minor damage to the vascular endothelium accompanied by adherence of platelets and leucocytes, leading to—

(II) massive infiltration by neutrophil polymorphs which increases vascular damage and impedes lymphatic drainage, causing oedema formation;

(III) delayed infiltration and local proliferation of mononuclear cells.

Stages (II) and (III) overlap, but are independent. Only (II) depends on circulating neutrophil polymorphs.

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THE MECHANISM OF ARTHUS REACTIONS. II. THE ROLE OF POLYMORPHONUCLEAR LEUCOCYTES AND PLATELETS IN REVERSED PASSIVE REACTIONS IN THE GUINEA-PIG.

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In the preceding paper (Humphrey, 1955) evidence was given for the importance of neutrophil polymorph accumulation in the development of vascular necrosis and oedema at sites of reversed passive Arthus (R.P.A.) reactions in the rabbit. It was desirable to ascertain whether the sequence of events was broadly similar in some other species, and the guinea-pig was chosen, both because its response to acute anaphylaxis is different from that of the rabbit, and because an excellent quantitative study of passive Arthus reactions in this species had already been made by Benacerraf and Kabat (1950). Exact repetition of the experiments made in rabbits was not possible because nitrogen mustard (HN2) proved to be too toxic to guinea-pigs when administered in doses sufficient to remove neutrophil polymorphs effectively from the circulation. Furthermore, no guinea-pig sera were available containing sufficient precipitating antibody to give easily measurable macroscopic reactions in R.P.A. reactions, and heterologous (rabbit) antisera had to be used instead. The first difficulty was overcome by the use of potent specific rabbit antibodies against guinea-pig polymorphs, following the work of Bedson (1921), and these proved to be a very effective means of removing mature polymorphs not only from the circulating blood, but also from the bone marrow. It was also possible to obtain potent specific rabbit antisera against guinea-pig platelets. By means of these various reagents it has been found that in the guinea-pig, as in the rabbit, polymorphs are essential for the formation of gross oedema at Arthus reaction sites, but not for the rapid anaphylactic increase in capillary permeability. In the absence of platelets, the severity of such reactions is somewhat increased.

METHODS.

Animals.

Albino guinea-pigs were used, weighing 350–550 g., from the stock bred at the National Institute for Medical Research. They were fed on pelleted Diet No. 18 (Bruce and Parkes, 1946) supplemented with cabbage and hay. The bellies were clipped and depilated on the day before testing.

Production of R.P.A. reactions.

Antigen was injected intraperitoneally, dissolved in 1 ml. 0·9 per cent NaCl. After 1–1½ hr. two intracutaneous injections of 0·2 ml. suitably diluted rabbit antiserum were made symmetrically into the belly wall on either side of the midline. Oedema volume was estimated from direct measurements of the swelling visible when the animal was held supine. The amounts of antibody in each injection were 1–2·5 mg., and the amounts of antigen were approximately 50 × the neutralising equivalents of the antibody. The antigen-

antibody systems were pneumococcus Type III capsular polysaccharide, 5 × recrystallised ovalbumin, crystallised bovine serum albumin (Armour), and human gamma globulin together with their respective rabbit antisera, prepared as described in the preceding paper. Most of the experiments were made with pneumococcus polysaccharide and the corresponding antiserum, but results were similar with the other systems.

It was impossible to test each animal before and after depletion of polymorphs, because the duration of depletion exceeded the period required for sensitisation to the antigens and antisera injected in the first test. Control groups of matched guinea-pigs were therefore included in each experiment.

Preparation of antisera against neutrophil polymorphs and platelets.

Neutrophil polymorphs were obtained from exudates induced by intraperitoneal injection of 50 ml. 3 per cent solution of bacteriological peptone in 0·9 per cent NaCl. After 15 hr. a further 20 ml. was injected intraperitoneally, and 3 hr. later the exudate, containing 90 per cent or more of polymorphs was collected, with addition of 1/5 vol. 3·8 per cent sodium citrate. The cells were washed 3 × with buffered Ringer solution containing 0·1 per cent gelatine, and were used fresh, or after storage for up to 3 days at 0°.

Platelets were obtained by differential centrifugation of blood taken by cardiac puncture from heparinised guinea-pigs, into silicone-treated tubes containing 1/10 vol. of 1 per cent di-sodium salt of ethylenediamine tetra-acetic acid (" Versene "—Bersworth Chemical Co., Framingham, Mass., U.S.A.). They were washed twice with 0·9 per cent NaCl solution at 2°, and suspended in buffered gelatine Ringer.

Rabbits were given intravenous injections of approximately 10^8 polymorphs or 10^9 platelets at 3-day intervals for a course of 3 weeks. Sometimes a second course was given 6–8 weeks later. Serum samples were tested at intervals for ability to agglutinate *in vitro* (in silicone-treated tubes) the cells against which the rabbits had been immunised. The end-point was taken as that dilution of serum which, after standing overnight at 4°, caused just definite macroscopic agglutination of polymorphs (5×10^6 per ml.) or platelets (5×10^8 per ml.). Sera with titres of at least 500 against polymorphs or 1,000 against platelets were heated at 56° for ½ hr. and kept for subsequent absorption. Both types of antisera contained agglutinins against erythrocytes, which were absorbed by successive treatments with washed erythrocytes, until only a very fine agglutination occurred. They were then treated with ½ vol. of washed liver cells, ¼ vol. of washed kidney cells, ¼ vol. of washed spleen cells, and the anti-platelet sera were finally absorbed with polymorphs obtained from peritoneal exudate. After absorption the globulins were precipitated twice with Na_2SO_4 (final concentration 18 per cent w/v) at pH 7·8, dialysed to remove Na_2SO_4, and made up with 0·9 per cent NaCl to a volume equal to the original volume of serum. About half of the original antibodies against polymorphs or platelets remained after the treatment outlined above, and they were specific for the cell types concerned.

Haematological examinations.

These were made on blood taken from ear veins after smearing with soft paraffin, and causing vasodilatation with toluene. In examinations for the presence or absence of platelets, the veins were pricked through sodium citrate solution.

RESULTS.

Effect of nitrogen mustard on blood neutrophils.

Although HN2 administration did not prove to be satisfactory, it is worth recording that a dose of 2 mg./kg. intravenously caused a marked reduction of polymorphs, beginning on the 4th day after administration and maximum on the 6th day (average count 100/c.mm.), with relatively little reduction in lymphocytes and monocytes. Doses of 1 mg./kg. were much less effective, while doses of 3 mg./kg. caused death after 4 days. There was thus no margin of safety when HN2 was used, and furthermore, even when polymorph counts were at their lowest, they increased 10-fold or more in response to injections of antigen.

Effect of antibodies on neutrophil polymorphs.

The effect of intraperitoneal injection of 2·0 ml./kg. anti-neutrophil globulins is illustrated in Fig. 1. The reduction in neutrophil polymorphs was rapid, and persisted for five days, in contrast to the delayed onset and transient nature of the neutropenia due to HN2 administration. Other formed elements of the blood (erythrocytes, lymphocytes and platelets) were unaffected, or—in the case of lymphocytes—suffered at most a 2–4-fold reduction. The animals showed no obvious effects from the injections, except that for two days afterwards the skin of the belly, exposed by depilation, was mildly erythematous.

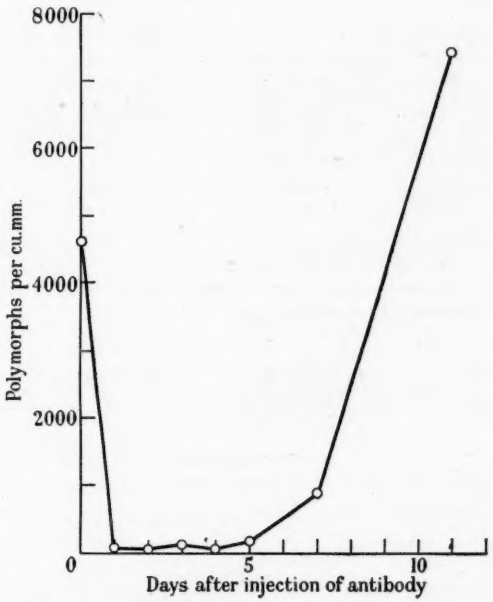


FIG. 1.—Effect of 2·0 ml./kg. anti-neutrophil globulins on neutrophil polymorph counts in peripheral blood of guinea-pig.

Of 46 guinea-pigs which received antibody, and whose differential blood count was done two days later, 22 had 0–1 per cent neutrophils, 16 had 2–4 per cent and 7 had 10 per cent or more. The neutropenia in the peripheral blood was accompanied by similar changes in the bone marrow, the percentage of mature neutrophils plus neutrophil myelocytes falling to between 9 and 20 per cent, instead of the normal 40 per cent or over. Guinea-pigs with neutropenia were very susceptible to bacterial infection, and it was found that strict sterile precautions were necessary where intravenous injections were made in the thigh, in order to avoid local cellulitis.

Effect of antibodies on platelets.

A single injection of 0·5 ml./kg. anti-platelet antibody globulins caused no immediate ill effects on the guinea-pig, but 24 hr. later the platelet counts had dropped to 20,000/c.mm. or lower. It was frequently impossible to detect any platelets in smears of peripheral blood, although leucocytes were unaffected. The animals had petechial haemorrhages in many parts of the body, particularly retroperitoneally and at sites of friction, and they became increasingly anaemic. Those which survived for 5 days recovered rapidly, and platelets returned to the circulation at this time.

Effect of neutropenia on oedema of R.P.A. reactions.

Reversed passive Arthus reactions were performed as described under "Methods" in guinea-pigs which had received 2·0 ml./kg. anti-neutrophil serum intraperitoneally two days previously, and in similar animals which had received corresponding amounts of normal rabbit globulins. An additional control was provided by animals of both series which were injected intracutaneously with rabbit antisera but did not receive antigen.

In the Table are recorded the measurements of gross oedema in treated and untreated animals. It is evident that in the absence of neutrophils oedema was minimal, as compared with the massive oedema which appeared in control animals. Despite this reduction in gross changes, however, neither the early increase in capillary permeability nor the later mononuclear cell invasion at the reaction sites was appreciably changed, as will be shown below.

TABLE.—*Mean Oedema Volumes at Sites of R.P.A. Reactions in Guinea-pigs with and without Neutrophil Polymorph Depletion.*

Treatment	Number of guinea-pigs.	Oedema vol. (ml.) after 7 hr.	24 hr.	48 hr.
Anti-polymorph antibody i.p.	9	0·4	0·5	*nil*
Normal globulin i.p.	11	1·4	3·0	2·6
Controls without antigen	16	0·3	0·13	*nil*

Each animal was injected at 2 sites.

Effect of neutropenia on changes in vascular permeability.

Reaction to histamine, 48/80 and leukotaxine.—Guinea-pigs were injected intraperitoneally with anti-neutrophil polymorph antibodies or with normal rabbit globulin. Two days later they were clipped and depilated, and were then injected intravenously with 0·6 ml./kg. 5 per cent Pontamine Sky Blue 6BX (G. Gurr). Immediately afterwards, 0·1 ml. quantities of 0·9 per cent NaCl containing graded doses of histamine (0·33–3 μg.), or the histamine liberator 48/80 (1–9 μg.) or leukotaxine (12–48 μg.) were injected intracutaneously, and the area and intensity of local blueing were noted. There were no differences between depleted and control animals.

Direct passive local anaphylaxis.—Guinea-pigs were sensitised passively by intraperitoneal injection of 1 mg./kg. rabbit antibody to pneumococcal capsular polysaccharide (Type III) at the same time as they received anti-neutrophil or

normal rabbit globulins. Two days later they were depilated, blued as above and straightway injected intracutaneously with 2 and 0·5 μg. of the polysaccharide in 0·1 ml. Blueing at the injection sites was rapid—being maximal in 10 min.—and was equal, both in area and intensity, in depleted and control animals.

Reversed passive local anaphylaxis.—In order to study the time course of the changes in capillary permeability, R.P.A. reactions were induced, as described above under "Methods", in 32 guinea-pigs of which half had been depleted of polymorphs in the usual way. Two doses of antibody, 1·0 and 0·04 mg., were used in separate experiments. Each animal received 4 intracutaneous injections of antibody, at different times relative to each other, and after a further interval Pontamine Blue was administered intravenously. In this way the distribution and intensity of capillary leakage was observed at intervals from a few minutes to 6 hr. after injecting antibody.

With the high doses of antibody blueing began around 30 min., and was maximal at 3½–5½ hr. During the later stages only the periphery of the lesions blued, the centres remaining pale, except for small central areas of haemorrhage. The pattern of blueing was similar to that described at sites of R.P.A. reactions in the rabbit (Humphrey, 1955), except that the reaction was more intense. There was no significant difference between the blueing reactions in polymorph-depleted and control animals.

With the low doses of antibody, the extent of blueing was much less, and there were no haemorrhages. Blueing began sooner, and was maximal at ½–1 hr. after injecting antibody. After 1½ hr. in normal guinea-pigs, the reaction site remained pale compared with the surrounding skin, whereas in polymorph-depleted animals increased capillary permeability was still evident up to 6 hr. It appeared that the closure of the vessels in the centre of these less intense lesions was in some way bound up with polymorph infiltration.

Effect of platelet depletion on R.P.A. reaction.

Six guinea-pigs depleted of platelets by injection of anti-platelet serum on the previous day, were subjected to R.P.A. reactions in the usual way. The reactions were compared with those in normal controls treated at the same time.

When judged by oedema formation (measured at 24 hr.) and haemorrhage, the reactions in the platelet-depleted animals were more severe than in the controls. Since the depleted animals had generalised petechial haemorrhages, strict comparison is impossible, but it was notable that much of the massive oedema at the reaction sites in these animals was not blood-stained—*i.e.*, it was true oedema fluid and not seepage from haemorrhages.

Histological changes at R.P.A. sites in normal and polymorph-depleted guinea-pigs.

Skin sites were excised 5, 24 and 48 hr. after the start of R.P.A. reactions in normal and polymorph-depleted guinea-pigs. Control sites, injected with normal rabbit serum or normal rabbit gamma globulin, were examined for comparison. They were stained with haematoxylin and eosin after fixation in corrosive sublimate and acetic acid.

The general picture—*i.e.*, oedema, vascular necrosis and thrombosis (with or without haemorrhage), perivascular infiltration by polymorphs and mononuclear cells—was similar in normal guinea-pigs to that in normal rabbits (see previous

paper), except in certain features. These were that already after 5 hr. the mononuclear cell accumulation was well marked; that oedema continued to increase and to persist for longer; and that polymorphs disappeared more slowly, being still present in very large numbers after 48 hr. In polymorph-depleted animals the histological picture was similar to that in normals as regards mononuclear cell accumulation, occurrence of platelet thrombosis, and oedema in the early stages; vascular necrosis, although present in all lesions to a variable degree, was less extensive than in normal animals, and at 24 and 48 hr. there was very much less oedema despite the marked mononuclear cell accumulation. These features are illustrated in Fig. 2–7.

DISCUSSION.

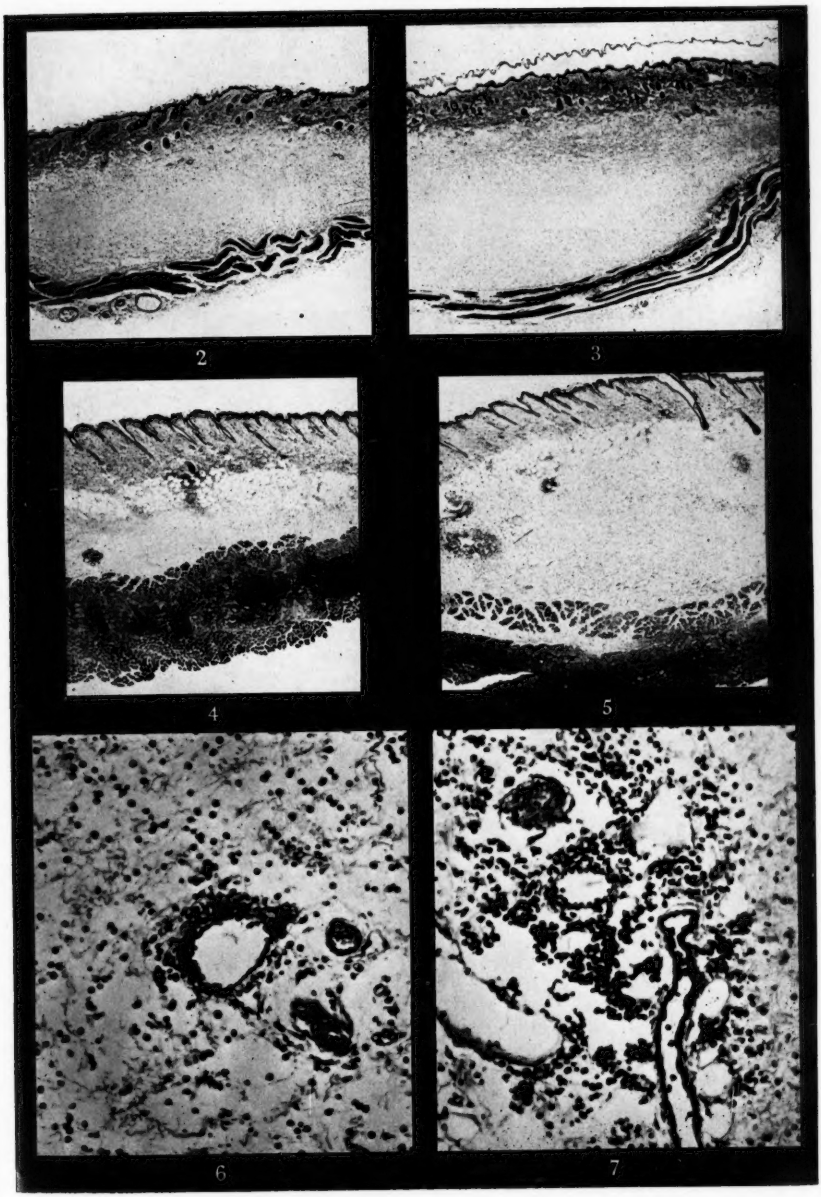
Fundamentally, the course of R.P.A. reactions in guinea-pigs and rabbits, and the effect of polymorph depletion on them, is similar. Since depletion was achieved by entirely different mechanisms, it seems that absence of polymorphs, rather than some other unknown factors, accounted for the marked diminution of oedema and the decrease in vascular damage observed in depleted animals. The conclusion reached in the previous paper, that polymorph accumulation was a major factor in exaggerating the damage to walls of capillary venules and, to a lesser extent, arterioles, and in blocking the lymphatics during the second stage of the reaction, is thereby substantiated. In the guinea-pig, in contrast with the rabbit, the increased vascular permeability of the earliest phase was very marked, and was uninfluenced by polymorph depletion. This may mean that guinea-pig skin vessels are relatively highly sensitive to the initial product(s) of the antigen antibody reaction, whereas in the rabbit the corresponding changes cause little more than increased endothelial stickiness. Differences of this nature could also explain the fact that whereas " passive cutaneous anaphylaxis " (Ovary and Bier, 1953) is readily elicited in guinea-pigs, no such reaction occurs in rabbits.

The more rapid accumulation of mononuclear cells at the reaction sites in guinea-pigs than in rabbits may be due to the use of heterologous antisera in the first species and of homologous antisera in the second. It could also be due to a species difference, and the experiments described do not allow of distinction between these possibilities.

Thrombosis being a prominent feature of severe or moderate Arthus reactions, it might be expected that prevention of thrombosis by platelet depletion would lessen the severity of the reaction—despite the fact that in rabbits large amounts of heparin were relatively ineffective. The severity was, however, actually increased, and platelets must, to some extent, play a protective rôle, either by causing thrombosis or by vasoconstriction (due to liberation of their contained 5-hydroxytryptamine), thereby limiting the area of inflammation.

EXPLANATION OF PLATES.

FIG. 2–7.—All show sites of reversed passive Arthus reactions in guinea-pig skin. Fig. 2: Polymorph-depleted; 5 hr. (× 18). Fig. 3: Normal guinea-pig; 5 hr. (× 18). Note greater oedema, and cellular invasion. Fig. 4: Polymorph-depleted; 2 days. (× 20). Perivascular cell collections are mononuclear cells. Fig. 5: Normal guinea-pig; 2 days. (× 18). Note persistent oedema. Perivascular cell collections are both mononuclear and polymorphonuclear. Fig. 6: Higher power of Fig. 2, showing early invasion by mononuclear cells in polymorph-depleted animal. (× 210). Fig. 7: Higher power of Fig. 5. (× 210).



Humphrey.

SUMMARY.

Guinea-pigs were deprived of blood neutrophil polymorphonuclear leucocytes and/or platelets by means of specific antisera.

Reversed passive Arthus reactions were studied in polymorph-depleted guinea-pigs and compared with those in normal animals. In the absence of polymorphs, oedema was very much diminished, polymorph invasion was absent, and vascular damage was decreased. Other changes, notably acute early increase in vascular permeability, and a relatively early mononuclear cell invasion, were unaffected.

The severity of the reactions was increased in animals without platelets.

The findings are compared with those in rabbits.

I am grateful to Professor A. A. Miles for discussion, advice, and help with some of the experiments described; to Mr. B. E. Price for technical assistance.

The compound 48/80 was supplied by Dr. E. J. de Beer of the Wellcome Research Laboratories, Tuckahoe, U.S.A.

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THE REACTIONS OF CERTAIN BACTERIOPHAGES WITH THEIR RECEPTORS ISOLATED FROM *SALMONELLA TYPHI* O 901 R.

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THE adsorption of a bacteriophage to its host cell is now considered to involve the formation of electrostatic bonds between the phage particles and specific receptor sites on the bacterial surface (Puck, 1953). It has been shown by Burnet (1934) and by Jesaitis and Goebel (1952) that it is possible to extract from bacterial cells material which can inactivate homologous phages and which is considered to contain the specific phage receptors. This assumption is based on the observation that the phage-inactivating power of the extracts is as specific as the phage-adsorbing power of the bacteria. For example a strain of *Shigella sonnei* which is lysed by phages T2, T3, T4, T6 and T7 yields on extraction a material which can inactivate all five of the phages, whereas the extract prepared from a variant which is resistant to T3, T4 and T7 inactivates only phages T2 and T6 (Goebel and Jesaitis, 1952).

The purified material from *Sh. sonnei* was found to be an antigenic complex of phosphorylated lipocarbohydrate and protein, but it could be degraded to give a serologically active protein-free lipocarbohydrate which still retained the ability to inactivate phages T3, T4 and T7. It had lost the power to inactivate phages T2 and T6. The action of the lipocarbohydrate on phage T4 was studied by Jesaitis and Goebel (1953) and it was found to result not only in the inactivation of the phage but also in the release of its nucleic acid into solution. This discovery is of great interest in the study of bacteriophage multiplication because it suggests that the phage receptor can be considered as the agent which causes the release of the phage nucleic acid at the bacterial surface ready for incorporation into the host cell.

The work described in this paper concerns the isolation from *Salmonella typhi* O 901 R of the receptors for some of the phages to which it is susceptible. The reaction of one of these phages with its receptor has been studied and found to result in the almost immediate release of nucleic acid.

MATERIALS AND METHODS.

Organisms.

The phage receptors were prepared from acetone-dried cells of *Salm. typhi* O 901 R, which is a "rough" variant of O 901. This organism is called "rough" because of its colony morphology, instability of suspensions in salt solutions and its susceptibility to an "anti-R" phage obtained from Dr. A. Felix, labelled "A.59/65.R". O 901 R is lysed by four phages in use in this laboratory, namely phage 1, phage 2, T3 and anti-R.

Stocks of bacteria for extraction purposes were grown in a medium containing acid hydrolysed casein 1 per cent, sodium lactate (syrup) 2 per cent v/v, sodium glycerophosphate

l per cent, tryptophan 0·0001 M and an aqueous extract of yeast 10 per cent v/v (Gladstone and Fildes, 1940). Batches of 1 l. were sterilised in 5–l. flasks, inoculated, and incubated at 37° in a spinning apparatus similar to that described by Mitchell (1949). After overnight growth the cells were collected and washed in 1 per cent NaCl by sedimentation. Suspensions of O 901 R in culture medium or saline are unstable and the cells settle to the bottom of the vessel in a few minutes. The concentrated cells were then centrifuged, suspended in water and precipitated with 6 vol. acetone. Several changes of acetone were used to dehydrate the cells which were finally filtered off and dried in air. The yield was between 3 and 4 g. of dried cells per l. medium.

Bacteriophages.

Four bacteriophages were used. These were T3, one of seven " T " coliphages, " anti-R " (Felix), a phage which attacks only " rough " strains of *Salm. typhi*, and phages 1 and 2. These latter were derived by single plaque isolations from a phage which attacked *Salm. typhi* N.C.T.C. 3390. Phage 2 is indistinguishable serologically from the " anti-O No. 2 " phage used by Dr. Felix (personal communication). Phage 1 has a different host range from phage 2 and is unrelated serologically. Stocks of phage T3 were prepared on *Bacterium coli* B grown in a defined medium containing 0·05 M phosphate, buffer pH 7·6, 0·02 M–NH_4Cl, 0·00016 M–$MgSO_4$ and 0·02 M glucose. Phages 1 and 2 were prepared by lysis of *Salm. typhi* R4 (derived from N.C.T.C. 3390) grown in the above medium supplemented with 0·00025 M–$CaCl_2$ which is an essential factor for the multiplication of these two phages. Phage anti-R was prepared by the lysis of R4 grown in tryptic meat broth supplemented with 0·002 M–$MgSO_4$. A high magnesium concentration is necessary for the adsorption of this phage to its host (Tucker, personal communication). All the lysates were centrifuged to remove bacterial débris and stored at 4° with the addition of chloroform to prevent bacterial contamination.

Radioactive phage 1 labelled with ^{32}P was prepared as follows: *Salm. typhi* R4 was grown in the defined medium modified by reduction of the phosphate concentration to 0·02 M and the addition of 10 μc./ml. of ^{32}P as orthophosphate.

Seven 10-ml. lots of this medium were rocked at 37° in " L " tubes (Kay, 1952) until the bacteria had grown to 4×10^8/ml. Phage 1 was then added to give a ratio of one phage particle per cell. Lysis was complete in 4 hr. and the phage was then separated and purified by three cycles of low (2000 g.) and high (30,000 g.) speed centrifugation. A concentrate containing $3·7 \times 10^{11}$ active phage particles per ml. with a ^{32}P radioactivity of 120,000 counts/ml./min. was obtained. High speed centrifugation sedimented 88 per cent of the radioactivity and a suspension of O 901 R cells in 0·03 M phosphate buffer was found to adsorb 98·5 per cent of the phage activity and 80 per cent of the radioactivity.

All four phages were assayed by the two-layer method on R4. Phages 1 and 2 were incubated at 37° overnight, but phages T3 and anti-R were ready for counting after 4–6 hr. Further incubation was undesirable because the plaques grew in size and coalesced.

Measurement of radioactivity.

Samples (0·1 ml.) were dried on aluminium dishes (2 cm. diameter) and counted under a thin window Geiger-Müller counter. When samples of larger volume had to be counted a liquid counter tube holding 10 ml. was used. At least 3000 counts were obtained from each sample.

EXPERIMENTAL.

Extraction of Phage Receptors from O 901 R.

A suspension of acetone-dried cells (50 g.) in water (300 ml.) was heated at 60° for 1 hr. with occasional shaking. After centrifugation the supernatant fluid was removed and the sedimented cells again extracted with water at 60° for 1 hr. Six serial extracts made in this way were clarified by centrifugation at 10,000 r.p.m. and assayed for phage-inactivating power by the following method. Serial tenfold dilutions of the extracts (2 ml.) in water were added to 0·067 M phosphate buffer at pH 7·6 (2 ml.) containing 0·004 M magnesium and 0·02 per cent gelatin. A

dilute suspension of the phage (0·1 ml.) was then added to each dilution of the extract and to a control tube containing buffer but no extract. The final phage concentration was about 1×10^3/ml. A sample from the control tube was assayed immediately to determine the initial phage titre. After incubation at 37° for 90 min. the phage titre in each tube was determined and the percentage of the phage particles inactivated was calculated. Phages 1, 2 and anti-R were tested in this way, but in order to test phage T3 the medium had to be modified to contain only 0·0002 M magnesium instead of 0·002 M. The magnesium concentration of 0·002 M had been chosen because it promoted the rapid inactivation of phage anti-R and was effective in preserving phage 1 from non-specific inactivation. Phage T3 however has little affinity either for its host bacteria or for its receptor in solution in the presence of 0·002 M-Mg, but at 0·0002 M Mg both reactions proceed at a satisfactory rate.

The percentage of phage 1 particles inactivated by different concentrations of each of the six extracts is shown in Table 1. A very approximate estimate of the

TABLE I.—*The Inactivation of Phage* 1 *by Six Serial Extracts of* Salm. typhi *O* 901 *R.*

Extract tested.	Concentration of extract in phage-extract mixture (μg./ml.).* 1000.	100.	10.	1.
1	66	27	22	0
2	99	53	0	0
3	100	79	22	0
4	100	93	43	20
5	100	96	23	0
6	100	93	71	0

* The figures represent per cent of total phage inactivated.

amount of receptor that inactivates 50 per cent of the phage was made by interpolation of a plot of the logarithm of the concentration against the percentage of phage inactivated. For example extract No. 1 inactivates 50 per cent of the phage at 400 μg./ml. but extract No. 4 is equally active at only 15 μg./ml. The " 50 per cent inactivation " concentration was calculated for each extract against all four phages. These figures obtained are shown in Table II, from which it can be seen

TABLE II.—*The Anti-phage Activity of Six Serial Extracts of* Salm. typhi *O* 901 *R against Four Strains of Phage.*

Extract tested.	Concentration of extract (μg./ml.) to inactivate 50 per cent of the phage. Phage 1.	Phage 2.	T 3.	Anti- R.
1	400	+	+	+
2	90	*	*	2,500
3	30	4,000	2,700	180
4	15	1,500	1,500	40
5	20	2,000	1,500	40
6	20	1,500	1,000	40

\+ No activity at highest concentration tested.
* Highest concentration caused less than 50 per cent inactivation.

that material active against phage 1 is found in all the extracts and against all four phages in the last five extracts. However, the activities against phage 1 and phage anti-R are much greater than those against phage 2 and phage T3. The weight of material in the extracts varied from 8·9 g. in the first to 0·66 g. in the sixth, and totalled 17·96 g. or 35·9 per cent of the weight of cells taken. In this series of extractions the sixth extract still contained anti-phage material and in another series the thirteenth was still active. Under the microscope the extracted cells appeared to be of normal bacillary shape and did not seem to have disintegrated to any marked extent. The low yield of phage 2 receptor can be explained by its lability to heat. A washed suspension of O 901 R in 0·033 M phosphate buffer was found to adsorb 50 per cent of phage 1 and 99 per cent of phage 2 in 40 min. In another suspension of bacteria which had been heated at 60° for 1 hr. the adsorption of phage 2 was reduced to less than 10 per cent, whereas that of phage 1 was almost unaffected at 49 per cent.

Purification of Phage Receptors.

Acid precipitation.

Following the procedure described by Jesaitis and Goebel (1952) an attempt was made to precipitate the phage receptors at an acid pH. Extracts 3, 4, 5 and 6 which contained most of the active material were pooled and adjusted to pH 4·2 with acetic acid after cooling to 4°. After 18 hr. the precipitate which formed was separated by centrifugation at 4° and dissolved in water by the addition of sufficient NaOH to bring the pH to 7·6. The supernatant was also adjusted to pH 7·6. Both fractions were dialysed against distilled water and tested for anti-phage activity. The supernatant was found to be inactive against phages 2, T3 and anti-R but slightly active against phage 1 (50 per cent inactivation required 1 mg./ml.). The precipitate was highly active against phage 1 (7 μg./mg.), but the activity against phage anti-R was reduced to one half. There was no activity against phage 2 and very little against phage T3. The lost activity against phages 2 and T3 could not be recovered by mixing the two fractions or adding the dialysate. It appears therefore that acid precipitation is only of value for concentrating the phage 1 receptor, as the receptors for phages anti-R, T3 and 2 are destroyed to a greater or lesser extent. At this stage work was restricted to the phage 1 receptor.

Precipitation with sodium chloride.

It was found that the phage 1 receptor was precipitated by the addition of 10 per cent NaCl. The calculated quantity of solid NaCl was dissolved in the cold solution (pH 7·6) of the acid-precipitated phage 1 receptor and stood at 4° overnight. The precipitate was removed by centrifugation and dissolved in water. After dialysis against distilled water the activity was found to have increased to a " 50 per cent inactivation " concentration of 2 μg./ml., an improvement of 3·5 times over the acid-precipitated fraction. The yield of this material was 230 mg. from 50 g. dried bacteria. It had a nitrogen content of 6·9 per cent.

Dissociation with phenol.

Jesaitis and Goebel (1952), working with the somatic antigen from *Sh. sonnei* which is active against phages T2, T3, T4, T6 and T7, found that treatment with

90 per cent phenol dissociated the antigen into protein and lipocarbohydrate parts. Activity against T3, T4 and T7 was retained in the lipocarbohydrate fraction, but phages T2 and T6 were no longer attacked. A sample of the NaCl-precipitated phage 1 receptor in solution was mixed with solid phenol until a solution containing 85 per cent w/v of phenol was obtained. This was stirred at room temperature for 30 min. and then dialysed until the phenol was removed. The dialysis sac then contained a precipitate which had a nitrogen content of 12·4 per cent and a solution containing material with a nitrogen content of 1·5 per cent. These materials were not active against phage 1 either alone or when mixed together.

A similar result was obtained with an antigen from O 901 kindly supplied by Dr. D. A. L. Davies. This material was produced by extraction of the cells with trichloracetic acid and was subsequently degraded into protein, carbohydrate and lipid fractions. When tested against phage 1 the complete antigen (250 μg./ml.) destroyed 94 per cent of the phage in 1 hr. but the degraded materials were all inactive.

The Inactivation of Phage 1 *by its Receptor.*

In order to study the release of nucleic acid from inactivated phage it was necessary to use an amount of phage which would contain sufficient nucleic acid to be measurable by the available techniques. It was therefore desirable to know the maximum amount of phage which could be inactivated by a given amount of receptor. After some preliminary experiments to decide the approximately equivalent quantities of phage and receptor the following experiment was carried out.

Phage 1 ($1{\cdot}2 \times 10^{11}$ particles) in 0·033 M phosphate buffer pH 7·6 (1·9 ml.) containing 0·002 M magnesium and 0·01 per cent gelatin was added to each of two tubes. Purified phage 1 receptor (0·1 ml. containing 325 μg.) was added to one tube and water (0·1 ml.) to the other (control). Both tubes were incubated at 37°. The phage titre in the control tube was determined at 0 and 20 min. and in the other at 5, 10 and 22 min. At 24 min. phage 1 ($1{\cdot}2 \times 10^{11}$ particles) was again added to each tube. Phage titres in the control were determined at 24 and 105 min. and in the tube containing the receptor at 31, 41, 46 and 110 min. The results in Fig. I show that the first addition of phage is almost completely inactivated (92 per cent), whereas the second was not damaged at all. The phage titre in the control tube remained constant within the experimental error. All the sites on the receptor to which phage 1 could adsorb must have been occupied by the first addition of phage, which appeared to have been in excess, since 8 per cent remained active. Under these conditions therefore 325 μg. of receptor were saturated by 92 per cent of $1{\cdot}2 \times 10^{11}$ particles of phage 1. In another test 325 μg. inactivated 82 per cent of $1{\cdot}5 \times 10^{11}$ particles. From the figures it can be calculated that one particle of phage 1 is inactivated by $2{\cdot}6\text{–}3{\cdot}0 \times 10^{-15}$ g. of receptor. Jesaitis and Goebel (1953) found that their lipocarbohydrate fraction from *Sh. sonnei* required only 2×10^{-16} g. to inactivate one particle of phage T4. However, the phage 1 receptor has received only two stages of purification whereas the *Shigella* material had been purified more rigorously and had been de-proteinised.

Release of nucleic acid from phage 1.

If the reaction between phage and its receptor releases nucleic acid which may then go into solution it should be possible to detect this by removing the phage

bodies by centrifugation and measuring the amount of nucleic acid left in solution. By the use of phage labelled with ^{32}P which is believed to go almost entirely into the nucleic acid it is possible to follow the release of nucleic acid into the medium in non-sedimentable form.

Radioactive phage 1 ($3{\cdot}7 \times 10^{11}$ particles) was incubated at 37° with 11·5 mg. purified receptor in 0·03 M phosphate buffer pH 7·6 (30 ml.) containing 0·002 M magnesium and 0·01 per cent albumin. A control of phage alone was also set up. Samples (5 ml.) were removed at intervals to determine the non-sedimentable ^{32}P. These were stored at 2° until all the samples had been collected and then centrifuged at 15,000 *g.* for 60 min. also at 2°. The supernatants were removed and assayed for ^{32}P. The non-sedimentable ^{32}P in the control was determined

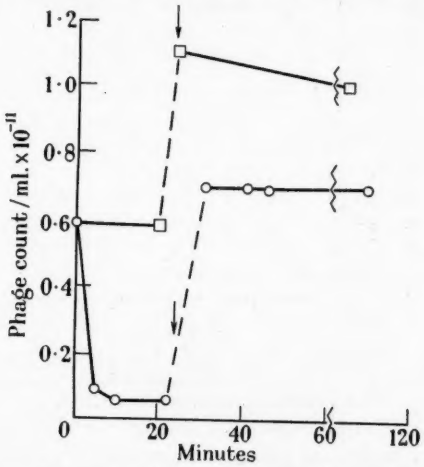

FIG. 1.—The inactivation of phage 1 in high concentration by purified receptor preparation (325 μg.). ⊡——⊡ Phage alone. ⊙——⊙ Phage plus receptor. Arrow indicates time of further addition of phage.

three times during the incubation period and found to be constant. The phage titres in both tubes were also determined. The storage of samples at 2° until a convenient time for centrifugation is justified since it was found that no ^{32}P is released for a period of 60 min. if the mixture of phage and receptor is kept at 2°. After making a correction for the amount of ^{32}P initially non-sedimentable the amount rendered non-sedimentable by the action of the receptor was calculated. Fig. 2 shows the result obtained together with a curve showing the inactivation of the phage. It can be seen that inactivation of the phage proceeds more rapidly than the release of ^{32}P especially in the first minute when 53 per cent of the phage is inactivated but only an insignificant amount of ^{32}P is found in the medium.

DISCUSSION.

The repeated extraction of O 901 R cells with water at 60° provides a satisfactory means of preparing the receptors for phages 1 and anti-R but not those for phages 2 and T3. The phage 2 receptor was found to be heat-labile as judged

by the lowered affinity for phage 2 caused by heating the cells at 60° and this could account for the low yield of anti-phage 2 activity in the extracts. The low yield of T3 receptor is unexplained. The action of the receptor in solution on phage 1 results in the rapid inactivation of the phage and a somewhat slower release of ^{32}P-containing material which may be phage deoxyribonucleic acid (DNA). The results of Jesaitis and Goebel (1953) showed that DNA was released when phage T4 was inactivated. Although there is a lag of about one minute's duration before a significant amount of ^{32}P appears in solution it is clear from the slope of the curve (Fig. 2) that ^{32}P and presumably DNA escapes from the phage

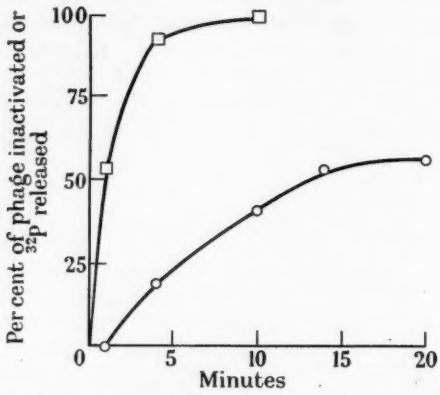

FIG. 2.—The liberation of ^{32}P from labelled phage 1 ($3 \cdot 7 \times 10^{11}$ particles) incubated with purified receptor (11·5 mg.). ⊙——⊙ Percentage of initially sedimentable ^{32}P rendered non-sedimentable. ⊡——⊡ Percentage of phage inactivated.

particles within a few minutes of their combination with the receptor. If the reaction between the phage and its receptor on the bacterial surface proceeds at a comparable rate then the first of the DNA will have left the phage particle and appeared on the bacterial surface in an equally short time. Whether the release of DNA at the bacterial surface is sufficient to constitute an active infection of the cell is unknown. It may be that an energy-requiring step then occurs which transfers the DNA across the cell wall. However the known properties of the phage receptor are sufficient to account for the specific adsorption of the phage particles to the host cell and the transfer of the DNA from the inside of the phage particle to the bacterial surface.

SUMMARY.

The receptors for three of the bacteriophages which attack *Salm. typhi* O 901 R can be isolated from the acetone-dried cells by extraction with water at 60°. The phages are inactivated by treatment with the receptor solution. Purification of the receptor material by precipitation with acid (pH 4·2) and NaCl (10 per cent) gave a product which was highly active against one of the phages (phage 1), one particle of which is destroyed by $2 \cdot 6 \times 10^{-15}$ g. of the receptor.

The inactivation of phage 1 results in the release into solution of material containing phosphorus which is presumably nucleic acid. The rate of release was

followed by the use of phage labelled with ^{32}P and found to be slower than the rate of inactivation.

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PATHOGENESIS OF FATTY AND SCLEROTIC LESIONS IN THE CARDIOVASCULAR SYSTEM OF CHOLINE-DEFICIENT RATS.*

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GROSSLY sclerotic aortic lesions and abnormal microscopic deposits of fat in coronary arteries have been observed in our laboratories in adult rats fed a low-choline diet for periods ranging from 5 to 7 months (Hartroft, Ridout, Sellers and Best, 1952). Subsequently we reported the rapid (2–7 weeks) development of fatty and necrotic lesions in cardiac muscle of young rats fed low-choline diets containing up to 40 per cent lard or beef fat (Wilgram and Hartroft, 1953; Wilgram, Hartroft and Best, 1954*a*). This observation confirmed and extended earlier findings of a somewhat related nature by Kesten, Salcedo and Stetten (1945), who found interstitial myocarditis in young rats fed choline-deficient diets containing 30 to 40 per cent ethyl laurate. Recently we have also reported (Wilgram, Hartroft and Best, 1954*b*) that young rats fed diets low in choline and high in naturally occurring fats, may develop within periods as brief as 17 days visible evidence of aortic sclerosis and microscopically demonstrable deposits of stainable fat in coronary arteries. The incidence of lesions in some of the latter experiments was as high as 90 per cent in the deficient animals. Controls given comparable amounts of the basal diet§ supplemented with choline chloride (0·85 per cent) failed to develop vascular abnormalities in any single instance. The incidence and severity of the vascular and cardiac lesion have been reported in these publications (*loc. cit.*). The purpose of the present paper is to describe and illustrate the pathogeny and pathology of the lesions and to report some recent experimental findings.

METHODS.

Details concerning preparation of the various diets, methods of housing and sacrificing the animals, and other information about the experimental procedures involved, have already been published in previous papers by the authors (Hartroft *et al.*, 1952; Wilgram *et al.*, 1954*a*, *b*).

Histochemical tests were performed on selected tissues to demonstrate cholesterol (Schultz, 1924), phospholipid (Baker, 1944), mucopolysaccharides (McManus, 1946), fatty acids (Fischler, 1904), ceroid (Wilson, 1950), haemosiderin (Perls, 1866) and calcium salts (von Kossa, 1901). The vascular systems of choline-deficient rats were injected with colloidal

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§ The percentage composition of the basal diet fed to young male rats (85–120 g.) was as follows: alcohol-extracted peanut meal 30, washed α-protein 5 (Glidden Co., Chicago, Ill.), casein 5, lard 35, P.D.W. vitamins 1, L.P. salts 3, cod liver oil concentrate 0·015, α-tocopherol acetate 0·01, sucrose 21.

suspension of carbon particles* to aid in the visualisation and study of the coronary vasculature. The method employed has been previously described by one of us (Hartroft, 1941) and utilises the preparation of thick cleared slices (200–500μ) as well as thin paraffin sections.

RESULTS.

Young rats fed severely hypolipotropic diets developed acute lesions in the heart and vessels within relatively short periods (sometimes less than 3 weeks), whereas older animals on less severely choline-deficient regimens did not show significant abnormalities in less than 3–4 months. The latter lesions were of a chronic nature and will therefore be described under a separate heading.

Acute lesions of heart and vessels.

Gross evidence of aortic sclerosis was not observed in any of the choline-deficient animals under a period of 17 days. Microscopic deposits of stainable fat, however, have been discovered in vessels (either aortas or coronary arteries) as early as 8 days after the initiation of the experimental regimen.

Fig. 1 illustrates initial alterations in a coronary artery of a young choline-deficient rat which developed the acute form of vascular lesion. Intimal endothelial cells are first affected. The cells become swollen and a diffuse sudanophilia affects the entire cytoplasm. Later, discrete droplets barely capable of resolution under the oil immersion objectives of the light microscope make their appearance. Still later (Fig. 2 and 3) stainable fat droplets are demonstrable in every layer of the arterial wall, including even the adventitia. Young rats fed severely hypolipotropic diets frequently died of either cardiac or renal cortical necrosis before the lesions in coronary arteries or aortas had progressed beyond the stage just described.

Frequently the fat may be more prominent in one layer than in others, and it appears that the distribution is governed largely not only by the amount of fat involved but also by the stage at which the lesion is observed. In the early phases of the disease the fat is in the inner portion of the vessel wall. Sometimes increased basophilia in the arterial wall has been noted (suggesting early and abnormal deposition of calcium salts), but in the acute coronary lesions we have not observed frank hyperplastic changes.

Abundant deposits of stainable fat in cardiac muscle cells (Fig. 4) can be demonstrated in a high percentage of the choline-deficient animals, but only rarely, and then in small amounts, in the controls. Fatty muscle cells subsequently became swollen, vacuolated and necrotic. Studies of the cardiac vasculature with the aid of ink injections showed (Fig. 5) that the muscle degeneration and necrosis were not ischaemic in origin, because capillaries in the regions of the necrosis were still patent. With necrosis and lysis of affected muscle cells, their fat droplets escape into the interstitium where they are engulfed by macrophages. In late stages of the lesion, areas of necrosis contain little fat. In the early stages of the inflammatory response to necrosis, polymorphonuclear leukocytes are prominent. At a later stage small round cells become more abundant, and with onset of healing fibroblasts appear and sarcolemmic cells proliferate at the periphery of the lesions.

* Higgin's India ink diluted with two parts distilled water and a few drops of ammonia added to ensure suspension and the whole filtered before use.

Frequently the distribution of muscle degeneration appeared to follow the localisation of the coronary arterial lesions, but this association was not found to be the case always.

In the aorta, intimal endothelial cells become sudanophilic in the initial stages of the lesion. Later there is degeneration and necrosis of the underlying media, associated with fatty change and eventual deposition of calcium salts (Fig. 6 and 7). Inflammatory reaction in the necrotic regions of the media is minimal or absent. The proliferative response in the intima is achieved by endothelial cells. Macrophages and foam cells were found only in very rare instances. In the late stages of the lesion (see below) intimal fibrosis may develop.

Chronic cardiovascular lesions.

Chronic changes were not observed in mature rats (180–200 g.) under a period of five months. The gross appearance of a typical lesion in an aorta is shown in Fig. 8. In the heart, minute flecks representing fibrosis could sometimes be observed with the aid of a hand-lens.

The coronary arteries showed eccentric intimal thickening in the form of small plaques which sometimes encroached on the lumen (Fig. 9). In these vessels, sites of lipid deposition were restricted to intima and media. In some instances the amount of lipid was less than that observed in the acute stages described above, suggesting that although the abnormal fat may have initiated the lesions, it had not remained *in situ* over the entire experimental period.

Fatty deposits in the myocardium in chronically choline-deficient rats were rarely encountered. Focal areas of myocardial fibrosis had developed, however, in a large number of the animals. The amount of collagen varied, and sometimes stained only palely with connective tissue stains. In many instances proliferated sarcolemmal cells were a more prominent feature than fibrosis *per se*. No constant pattern of distribution for the fibrotic changes could be discerned.

The microscopic features of the aortas in the rats subjected to chronic choline deficiency were not essentially different from those of the late stages of the acute changes described above. In many instances fractures of the medial bars of calcification were encountered, sometimes in various stages of callus formation and healing. In the most advanced cases, the entire thoracic aorta was converted into a rigid dilated pipe.

Histochemistry of hearts and aortas.

The lipid deposits were subjected to the series of histochemical tests recommended by Lison (1953) for the identification of deposits of sudanophilic fat in sections. In the acute lesions the lipid was positive to tests for neutral fat and fatty acids, and negative to tests for phospholipid, cholesterol and cholesterol esters. Deposits of calcium salts were present in acute lesions of both hearts and arteries, including aortas. Tests for ceroid were uniformly negative.

PAS (McManus) stains on hearts and aortas did not yield any significant increase of mucopolysaccharides in the necrotic areas of the cardiac muscle, but in aorta and coronary arteries, where the choline-deficient regimen led to hyperplasia of the inner layer of these vessels, mucopolysaccharides showed up intercellularly in the form of diffuse plaques as well as subendothelial nodules (Fig. 9 and 10).

Iron.—Of interest was a positive haemosiderin reaction throughout the entire

wall of some of the coronary arteries and within the inner layer of the aorta in acute and chronic choline-deficient experiments (Fig. 11 and 12). The Prussian blue reaction appeared rather diffusely throughout and no red blood cells or débris from their breakdown were to be found in the tissues specified above.

Frequently iron pigment also appeared in granular form within the cyto-architecture of many intact cardiac muscle fibres. Similar pictures have been seen in heart muscle and coronary vessels in haemochromatosis (Granick, 1954), although the mechanism of iron deposition in this latter disease is likely to be different from that operating in our experiments. There is no grossly visible haemolysis in our choline-deficient animals, and whether a bone-marrow depressed by the impaired kidney in choline deficiency cannot utilise the amount of iron offered, or whether the fatty choline-deficient liver cannot store iron in sufficient amounts and the surplus is partly deposited in cardiac muscle and coronary arteries, awaits further investigation. Iron balance studies might show if changes of the " intestinal mucosal block " are responsible for an increased iron absorption with subsequent deposition of iron in the cardiovascular system. Careful laboratory investigation was able to rule out artefacts in staining technique (calcium, formol haematin).

It is interesting that Salmon and Copeland (1954) described an increase in iron-positive pigment in the liver and kidney of choline-deficient chickens.

Lesions in organs other than hearts and aortas.

The pathology of the hepatic, renal, testicular and ocular lesions in choline-deficient rats has been described elsewhere (Hartroft, 1948, 1950 ; Hartroft and Burns, 1949 ; Hartroft and Ridout, 1951). The young animals almost consistently showed the classical gross and microscopic lesions of the haemorrhagic renal syndrome first described by Griffith and Wade (1939). The possible relation between the acute cardiovascular damage and these changes in other organs, especially those in the kidneys, will be discussed.

Experimental Variations.

Hormones.—Female rats are very resistant to the induction of cardiovasculorenal lesions in choline deficiency. However, by the simultaneous administration of androgens (5 mg./150 g. body wt.) and growth hormone (0·1 mg./150 g.) it is possible to produce renal and cardiovascular damage as described above in 80 per cent of females.

Cholesterol.—Firstbrook (1950), Moses and Longabaugh (1950) and Duff and Meissner (1951) have all demonstrated that the addition of choline to *normal* diets of rabbits given high doses of cholesterol has no inhibitory effect on the development of atheromatous changes. A surplus of choline has therefore no beneficial effect on this type of vascular degeneration. It should be noted that the amounts of cholesterol employed in the experiments of these authors were large and the basal diets already contained abundant lipotropic factors.

Our choline-deficient diets which were routinely employed for the production of cardiovascular disease contained no added cholesterol and the constituents in the diet were relatively free of this lipid. Cholesterol serum levels in choline-deficient rats were lower than in the choline-supplemented controls (Ridout,

Patterson, Lucas and Best, 1954). However, cholesterol supplements given to choline-deficient rats, which were otherwise resistant to the induction of cardiovasculorenal lesions (female rats, older males) produced the changes in 75 per cent of the animals. Cholesterol given to choline-supplemented rats had no ill effect whatsoever, although the cholesterol serum levels were again higher than in the cholesterol-supplemented choline-deficient group. All animals were pair-fed. Thus it appears that dietary cholesterol aggravates the effects of choline deficiency on the cardiovasculorenal system of choline-deficient rats, although it is by no means essential for the production of these lesions. This aggravating effect of cholesterol on the lesions (liver, kidney, heart and vessels) is not accompanied by an elevation of serum cholesterol.

DISCUSSION.

Pathogenesis.—Observations indicate that cardiovascular degeneration in choline-deficient rats is initiated by the appearance of abnormal deposits of stainable fat at the sites of the lesions wherever they may be. In heart muscle they are followed by swelling of affected myocardial cells with their eventual necrosis, lysis and replacement by fibrosis. In coronary arteries, fatty intimal cells may eventually undergo eccentric hyperplasia; the media may thicken concentrically and the supporting fibrous tissue in the adventitia may proliferate, particularly in mature animals with chronic lesions. In the aorta, intimal hyperplasia of fatty endothelial cells develops, accompanied by medial degeneration, necrosis and calcification. The mid-portion of the media is most frequently affected and we have previously presented evidence to suggest that interference with the nutrition of elasto-muscular tissue deep in the aortic wall may be one of the responsible factors (Hartroft *et al.*, 1952). The concept that abnormal deposits of fat in parenchymal cells may eventually lead to irreversible structural lesions has been proposed as a cause of the characteristic change in livers and kidneys of choline-deficient rats. In the liver, over-distension of hepatic cells by fat leads to cirrhosis (Hartroft, 1950), and in the kidney abnormal lipid precedes either bilateral cortical necrosis or atrophic regression of tubules (Hartroft, 1948). The evidence suggests that there may be but few sites in the body other than adipose tissue, wherein appreciable degrees of fat storage may occur for long without damaging the tissues or organs involved. We do not know why abnormal fatty deposits should be harmful to the cells in which they accumulate. In the cardiovascular lesions in choline-deficient rats, abnormal fat deposits certainly constitute the initial stage in their pathogenesis.

Aetiology.—Elsewhere we have discussed the evidence concerning the aetiology of the initial fatty deposits (Wilgram *et al.*, 1954*a*, *b*). Cardiac necrosis is probably independent of renal damage and is a direct effect of choline deficiency on heart muscle of young rats, because if the animals are fed diets containing ethyl laurate or trilaurine as the source of fat, massive cardiac necrosis may prove fatal before renal damage has developed. The relation between vascular and renal damage is not yet clear. Renal lesions produced by surgical methods in dogs (McCormick and Holman, 1949) or nephrotoxic agents administered to rats (Wissler, Collins, Schroeder and Soules, 1953; Lehr and Churg, 1952) are associated with vascular lesions not unlike those we have seen in choline-deficient rats. But the lipid component of the vascular lesions produced directly by renal damage is not as prominent as in those vessel changes we have encountered in choline deficiency.

Furthermore, Copeland (1954, personal communication) has observed vascular lesions in choline-deficient chickens and dogs identical with those in the rats, but unassociated with significant degrees of renal abnormalities. Artom (1954) has found that choline injected into choline-deficient rats a few hours before removing slices of their livers, kidneys and hearts enhances the rate of fatty acid oxidation in these tissues, compared with non-injected choline-deficient controls similarly observed. These observations of Copeland and of Artom taken together suggest that the cardiac and coronary damage in hypolipotropic rats may eventually prove to be a direct result of insufficient supplies of dietary choline and its precursors. The aortic lesions however, seem in part at least to depend on renal damage. The choline-deficient kidneys may be impaired in their capacity to excrete calcium and phosphorus and the retention of these minerals may conceivably lead to metastatic calcifications. We feel at present that this mechanism, known clinically as secondary hyperparathyroidism in chronic renal disease, together with choline deficiency *per se* as an equally important factor, may be responsible for the development of aortic lesions in choline-deficient rats. On the other hand, even if vascular lesions in our rats should prove to be entirely secondary to the renal damage induced by choline deficiency, the experiments may be of some interest in that the whole lesion-complex has been produced by brief periods of a *dietary deficiency alone*, rather than by the introduction of toxic agents or by surgical manipulations.

SUMMARY.

The pathology and pathogenesis of cardiovascular lesions in choline-deficient rats of various ages are described. Coronary arterial lipoidosis, grossly evident aortic sclerosis and myocardial necrosis have been observed in both acute and chronic forms. Choline supplements added to the basal diet prevented the development of comparable lesions in controls. Deposits of stainable fat initiated the induction of cardiovascular lesions by diets low in choline.

The sequence of changes in hearts and arteries is described. Lipid appeared within a few days in cardiac muscle cells that subsequently rapidly underwent swelling and necrosis. Intimal fat in coronary arteries eventually led to eccentric hyperplasia of endothelium and luminal narrowing. In the aorta, fatty deposits in endothelial cells were followed by formation of intimal plaques. The underlying media became necrotic and calcified.

There is some evidence that cardiac muscle necrosis and coronary lipoidosis may be independent of renal damage induced in rats by choline deficiency. Aortic lesions may be secondary to the kidney changes, but the possibility has not been excluded that choline deficiency is also directly implicated in the aortic lesions.

A positive haemosiderin reaction was obtained in the walls of coronary arteries and aortas as well as within intact cardiac muscle fibres. The reaction was diffuse throughout the vascular tissues and granular in the cardiac muscle. It was not dependent on local lysis of erythrocytes or artefacts. Its explanation is not yet known.

Androgen and growth hormone, administered simultaneously, induced cardiovasculorenal lesions in choline-deficient animals which were otherwise resistant to their development.

The addition of cholesterol to the choline-deficient diet greatly aggravated the ill effects of choline deficiency on the cardiovasculorenal system, without any accompanying elevation of serum cholesterol. Cholesterol given to choline-supplemented controls had no ill effect.

The clinical significance of these results is not apparent, but the rapid production of cardiovascular lesions in rats by a dietary deficiency alone may offer another useful experimental approach to the problems of coronary arterial disease and arteriosclerosis in man.

The authors are indebted to Professor C. H. Best for his guidance and advice throughout the entire course of this project. They are grateful to Dr. Jessie H. Ridout for her help and assistance in preparing and devising the diets. They acknowledge with thanks the aid of Mr. W. D. Wilson for cutting and staining the many hundreds of histological sections, and appreciate Mrs. M. Cornell's and Mrs. M. E. Lindsay's assistance in preparing the manuscript.

EXPLANATION OF PLATES.

FIG. 1.—Initial alterations in a coronary artery of a young choline-deficient rat are shown. Tiny fat droplets (black in photograph) appear in the intima, the cells of which are swollen and weakly sudanophilic. Frozen section, Oil Red O stain. × 300.

FIG. 2.—Lipoidosis is more advanced in this coronary artery of a choline-deficient rat than that illustrated in Fig. 1. The intima is swollen and strongly sudanophilic. Fat droplets and globules appear in the media and even in the adventitia. Frozen section, Oil Red O stain. × 350.

FIG. 3.—In this advanced lesion in the coronary artery of a young choline-deficient rat large masses of lipid appear throughout the entire wall of the blood vessel. Frozen section, Oil Red O stain. × 350.

FIG. 4.—Stainable fat is deposited in cardiac muscle fibres in an area of acute necrosis. The high-power inset shows the fat droplets arranged in the pattern of the cardiac muscle striation. Frozen section, Oil Red O stain. × 30, × 280.

FIG. 5.—India ink was injected into the coronary arteries of this young choline-deficient rat. The cardiac muscle fibres are swollen, vacuolated and necrotic, but the capillaries in this area are still patent to the injection fluid. Haematoxylin-eosin stain, × 400.

FIG. 6.—Fat is deposited (early stage) within the medial layer of the aorta of a young rat fed a severely hypolipotropic diet. The site of this lesion is near the mouth of one of the two coronary arteries. Frozen section, Oil Red O stain, × 300.

FIG. 7.—In later stages of aortic lesions in young choline-deficient rats as shown here, fat deposition has largely been replaced by gross calcification mainly located in the media of the aortic wall. Frozen section, Oil Red O stain, × 35.

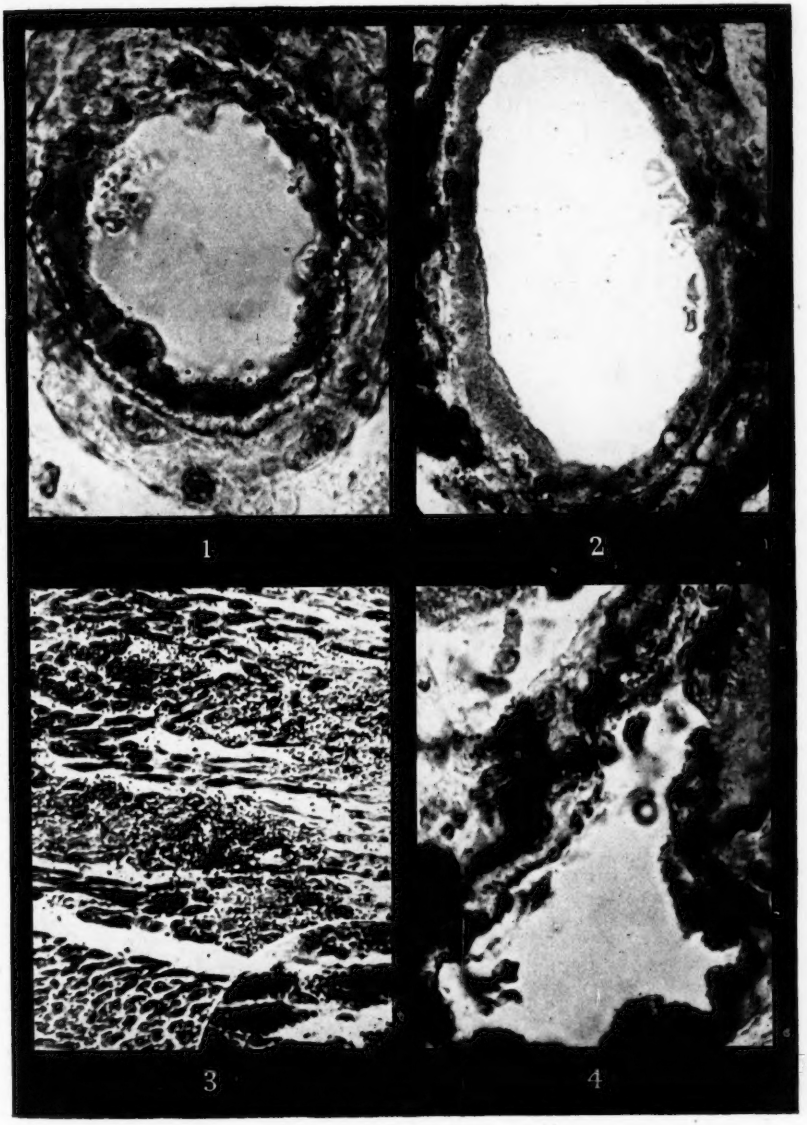
FIG. 8.—This aorta of a mature choline-deficient animal resembles a stick of bamboo because of medial sclerosis.

FIG. 9.—Eccentric intimal thickening and plaque formation has occurred in this coronary artery of a mature rat, suffering chronic choline deficiency. A subendothelial nodule is well demonstrated by this stain for mucopolysaccharides, (PAS). × 320.

FIG. 10.—Subendothelial fibrosis, hyperplasia and diffuse medial swelling of an aorta of a chronically choline-deficient animal is illustrated. The intercellular substance shows consolidation and separates the medial layers. Periodic acid stain, × 100.

FIG. 11.—Haemosiderin is deposited in coarse granules within intact cardiac muscle fibres in a young choline-deficient animal. There is no red cell breakdown visible in this section. Haemosiderin stain, × 1000.

FIG. 12.—The entire intima and media of this coronary artery of a mature choline-deficient rat stain diffusely throughout. Artefacts or post-mortem changes have been excluded. No erythrocyte breakdown is apparent in this section, stained by the Prussian blue reaction for haemosiderin, × 350.



Wilgram and Hartroft.

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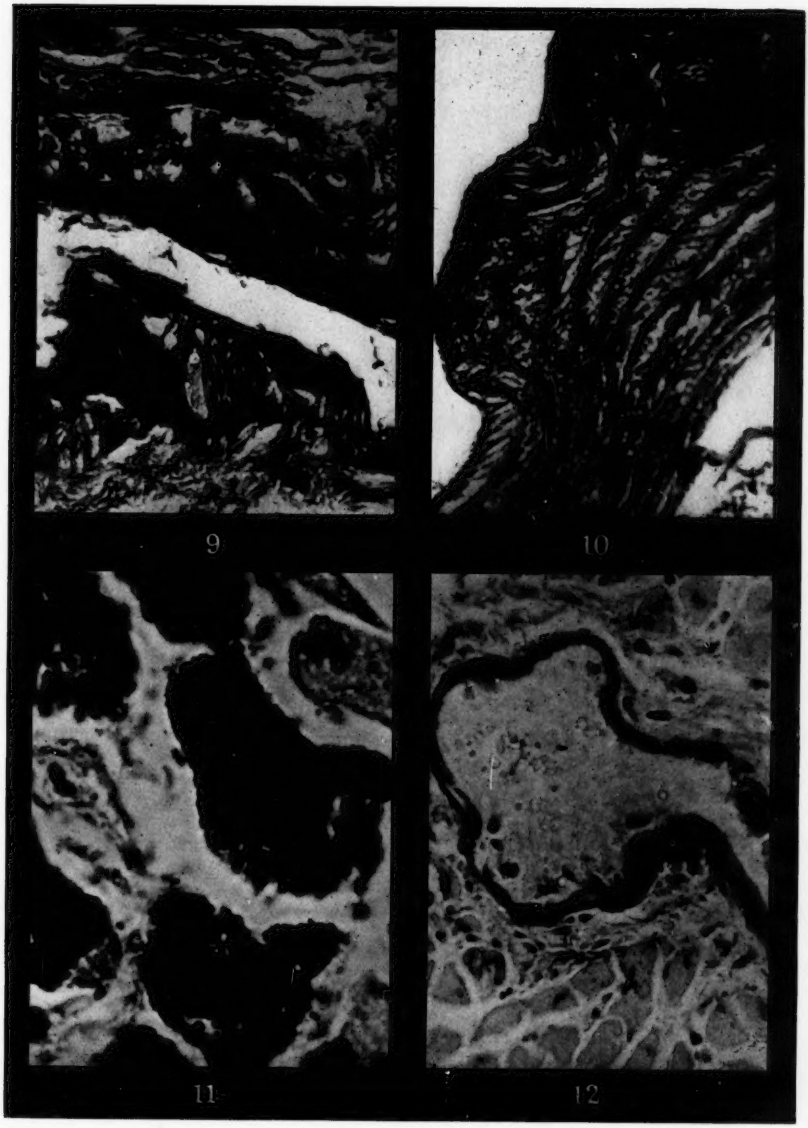

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STUDIES ON THE MECHANISM OF SHOCK.
THE EFFECT OF LIMB ISCHAEMIA ON PHOSPHOMONOESTERASE DISTRIBUTION.

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In recent papers on anthrax in the guinea-pig Smith and his co-workers have shown (Smith, Keppie, Ross and Stanley, 1954; Smith, Keppie, Stanley and Harris-Smith, 1955; Ross, 1955) that fatal infection with *Bacillus anthracis* is associated with a shock-like condition indistinguishable from that produced by other injuries. In addition to the usual changes in blood chemistry seen after severe injury, they found that the acid and alkaline phosphomonoesterase (phosphatase) activities of the plasma and urine were increased. From histochemical studies it was thought that this was due to the liberation of these enzymes by the damaged kidney, *B. anthracis* having been previously excluded as a possible source.

As very few changes in enzyme activity have been demonstrated in shock it was of interest to determine whether this was a general effect of injury or specific to *B. anthracis* infection. Available evidence (Herlant and Timiras, 1950; Valentine, Follette, Hardin, Beck and Lawrence, 1954) suggested the former and experiments were undertaken to see if these changes occurred after another form of severe injury—hind limb ischaemia. From the results to be described increased plasma phosphatase activity appears to be part of the general response to injury.

METHODS.

Rabbits, rather than rats or mice, were chosen for these experiments as their blood shows less haemolysis after muscle injury. Adult males were used fed on M.R.C. diet 18 (Bruce, 1947) and given access to food and water until the start of the experiment (10.0 a.m.). A 4-hr. period of bilateral hind-limb ischaemia was produced by the method of Bywaters and Popják (1942). This was nearly always fatal, the survival time (about 2·5 hr.) being the same as in a previous series (Stoner, 1954). The general features of the response to this injury are described there and in a previous paper (Whiteley, Stoner and Threlfall, 1953). In the nephrectomised animals the kidneys were removed under ether anaesthesia through separate dorsal incisions on the afternoon before the experiment. Samples (4·0 ml.) of blood were removed by cardiac puncture and heparin was used as an anticoagulant. Larger blood samples were taken when the properties of the enzymes were being studied. Control animals were submitted to a period of ether anaesthesia equivalent in depth and duration to that used for the application of the tourniquets. The initial blood samples were taken before the anaesthesia and the others at intervals afterwards so that, as far as possible, three samples were taken from each rabbit during the experiment. In some cases, where death from haemopericardium occurred after the second heart puncture or the rabbit died very soon after release of the tourniquets, only two samples could be obtained. After limb ischaemia the final sample was usually taken while the rabbit was dying.

The acid and alkaline phosphatases of the plasma were determined, using disodium phenyl phosphate as substrate, by the method of King (1951) and the results expressed in his units. An exception to this was for the determination of pH optima when the two separate buffers were replaced by B.D.H. universal buffer (Prideaux and Ward, 1924). For the phosphatase activity of the liver and kidney, samples (approx. 5 g.) were ground with distilled water in an Atomix Blendor (M.S.E.) to give a 1 per cent suspension which was substituted for plasma in the King method and $MgSO_4$ (final conc. 0·01 M) added as an activator (Aldridge, Barnes and Denz, 1950). The results are expressed as mg. phenol liberated from disodium phenyl phosphate by 100 g. wet wt. of tissue during 15 min. incubation at 37°.

The alkaline phosphatase activity of the kidney and liver was studied histochemically on frozen sections (10μ) according to Pearse's (1953) modification of the methods of Menten, Junge and Green (1944) and Manheimer and Seligman (1948), using α-naphthyl phosphate (Gurr) as substrate and the stable diazotate of 4-chloro-*o*-anisidine (Gurr). Plasma Mg concentration was determined by the method of Garner (1946). Colour comparisons were made with a Coleman "Junior" spectrophotometer. The statistical significance of the results was assessed by analysis of variance (Fisher, 1935) considering the differences between rabbits and between samples in those rabbits which contributed three blood samples. The Tables include the means with their standard errors (S.E.) determined from the residual mean square in the analysis of variance.

TABLE I.—*Effect of Short Ether Anaesthesia and Repeated Cardiac Puncture on the Alkaline Phosphatase Activity of the Plasma of the Normal and Nephrectomised Rabbit.*

Rabbit no.	Initial level.	Phosphatase activity (units/100 ml. plasma) Time (hr.) after anaesthesia. 4.	5.	6.	7.
138	8·2	7·7	—	6·8	—
142	7·3	8·8	—	7·3	—
146	11·4	11·6	—	12·1	—
163	5·3	5·3	5·2	—	—
164	4·2	3·8	—	3·1	—
178	9·3	10·4	—	10·3	—
179	11·7	—	11·7	—	10·9
180	9·8	—	10·8	10·7	—
181	8·7	—	9·7	9·7	—
Mean	8·4	7·9	9·4	8·6	—
S.E.*	0·17	0·22	0·27	0·21	—
176†	7·1	—	8·7	—	6·3
192†	7·6	—	8·0	—	8·4
194†	10·2	—	8·7	—	8·0
Mean	8·3	—	8·5	—	7·6
S.E.*	0·61	—	0·61	—	0·61

* = Standard error calculated from "residual" mean square in analysis of variance.
† = Bilaterally nephrectomised.

RESULTS.

Control Rabbits. Effect of Anaesthesia and Cardiac Puncture on Plasma Phosphatase Activity.

In the control rabbits a short period of ether anaesthesia and the removal of three blood samples by cardiac puncture did not affect either the alkaline or acid phosphatase activity of the plasma (Tables I and II). The mean level of activity did not differ significantly from the initial level at any of the times subsequent to anaesthesia at which observations were made although there were significant

differences ($P < 0.001$) between the mean levels in different individuals. This pattern was not altered by nephrectomy.

TABLE II.—*Effect of Short Ether Anaesthesia and Repeated Cardiac Puncture on the Acid Phosphatase Activity of the Plasma of the Normal and Nephrectomised Rabbit.*

Rabbit no.	Initial level.	Phosphatase activity (units/100 ml. plasma) Time (hr.) after anaesthesia. 4.	5.	6.	7.
138	16·2	13·0	—	11·1	—
142	8·4	11·2	—	7·8	—
146	19·8	13·8	—	16·0	—
163	4·3	3·6	3·4	—	—
164	5·1	4·2	—	3·9	—
178	16·4	19·2	—	16·8	—
179	15·8	—	12·5	—	12·9
180	18·5	—	18·8	18·7	—
181	25·0	—	26·7	24·5	—
Mean	14·4	10·8	15·3	14·1	—
S.E.*	0·54	0·66	0·81	0·61	—
176†	15·3	—	15·0	—	16·4
192†	16·3	—	15·7	—	18·6
194†	8·0	—	7·0	—	7·5
Mean	13·2	—	12·6	—	14·2
S.E.*	0·44	—	0·44	—	0·44

* = Standard error calculated from " residual " mean square in analysis of variance.
† = Bilaterally nephrectomised.

Effect of Limb Ischaemia on Plasma Phosphatase Activity.

In rabbits exposed to a 4-hr. period of bilateral hind limb ischaemia the alkaline phosphatase activity of the plasma rose progressively and in rabbits where the final blood sample was taken as the animal died from the effects of the muscle ischaemia it was an average of 187 per cent above the initial level (Table III).

TABLE III.—*Effect of a 4-Hour Period of Bilateral Hind Limb Ischaemia on the Alkaline Phosphatase Activity of the Plasma of the Rabbit.*

Rabbit no.	Initial level.	Phosphatase activity (units/100 ml. plasma). Before release of tourniquets.	Time (hr.) after period of ischaemia. 0·5.	1.	2.	3.	4.
141	4·2	4·8	—	—	9·0	—	—
143	5·5	—	7·8	—	—	—	14·9D
145	5·2	6·2	—	12·2D	—	—	—
157	3·8	4·3	—	—	—	8·1	—
166	11·1	—	—	23·3	33·0D	—	—
168	3·7	—	—	3·8	—	16·8D	—
182	10·3	—	12·2	—	—	20·9	—
183	8·3	—	14·5	—	—	—	28·3D
185	12·1	—	—	18·9	—	27·9D	—
189	5·6	—	—	5·7	—	9·9D	—
191	8·6	—	—	13·4	—	12·4	—
Mean	7·1	5·1	11·5	12·9	21·0	16·0	21·6
S.E.*	1·07	2·04	2·04	1·45	2·51	1·45	2·51

* = Standard error calculated from " residual " mean square in analysis of variance.
D = Death due to limb ischaemia.

Similar, though less striking, changes occurred in the acid phosphatase activity of the plasma (Table IV). The mean percentage increase at death in this case was 63.

In both cases the increase was statistically significant although the level of significance was greater ($P < 0.001$) for the alkaline than the acid phosphatase ($P < 0.01$).

One rabbit survived the ischaemic injury and showed no change in the alkaline or acid phosphatase activity of the plasma during the 24 hr. of the experiment.

TABLE IV.—*Effect of a 4-Hour Period of Bilateral Hind Limb Ischaemia on the Acid Phosphatase Activity of the Plasma of the Rabbit.*

		Phosphatase activity (units/100 ml. plasma).					
			Time (hr.) after period of ischaemia.				
Rabbit no.	Initial level.	Before release of tourniquets.	0·5.	1.	2.	3.	4.
141	8·1	7·0	—	—	9·2	—	—
143	9·4	—	10·9	—	—	—	17·8D
145	8·3	9·2	—	13·4D	—	—	—
157	7·5	12·8	—	—	—	8·4	—
166	22·8	—	—	23·8	31·5D	—	—
168	8·1	—	—	8·7	—	20·0D	—
182	23·4	—	24·8	—	—	20·7	—
183	14·8	—	18·8	—	—	—	25·1D
185	24·5	—	—	24·3	—	25·9D	—
189	21·2	—	—	21·4	—	27·3D	—
191	3·1	—	—	5·7	—	7·5	—
Mean	13·7	9·6	18·2	16·2	20·4	18·3	21·5
S.E.*	0·85	1·62	1·62	1·15	1·98	1·15	1·98

* = Standard error calculated from " residual " mean square in analysis of variance.
D = Death due to limb ischaemia.

Properties of the Plasma Phosphatases in Normal and Post-ischaemic Rabbits.

The pH optima (4·5 and 10·5) of the plasma phosphatases were unaltered after hind limb ischaemia. The addition of formaldehyde to the reaction mixture (Abul-Fadl and King, 1949 ; King, 1951) affected the control and post-ischaemic plasma similarly. The alkaline phosphatase was almost completely inhibited but there was only slight (13 per cent) inhibition of the acid phosphatase.

Seitz filtration (Ford's Sterimats S.B.) of the plasma had an interesting effect. The alkaline phosphatase activity was unaltered by this procedure which markedly reduced the acid phosphatase activity especially after limb ischaemia. The average percentage loss in acid phosphatase activity on passing control plasma through a Seitz filter was 29 per cent. Plasma collected after hind limb ischaemia lost 59 per cent of its acid phosphatase activity when treated in this way. The increase in the acid phosphatase of the plasma after injury could be largely removed by Seitz filtration. Millipore filters (Lovell Chemical Co., Mass., U.S.A.) of the type routinely used by Smith *et al.* (1955) to free the plasma from bacteria had much less effect on the acid phosphatase activity of the post-ischaemic plasma.

Mg^{2+} is an activator of phosphatases (Kay, 1932) and it was found that pre-incubation of the plasma with disodium ethylene diamine tetra-acetate (final conc. 0·098 M) at room temperature caused a 50 per cent reduction in alkaline

phosphatase activity while leaving the acid phosphatase activity unchanged. The changes in alkaline phosphatase activity might be due to alterations in the plasma Mg content. Previous work (Stoner and Green, 1949) has shown the plasma Mg level to be raised after hind limb ischaemia. In the present experiments the mean plasma Mg levels (mg. per 100 ml. $\pm$ standard deviation) in 8 normal rabbits after hind limb ischaemia (6·34 $\pm$ 1·08), in 9 nephrectomised rabbits after hind limb ischaemia (6·58 $\pm$ 1·65) and in 15 nephrectomised control rabbits (5·28 $\pm$ 1·76) were all significantly greater ($P < 0.001$) than in 15 normal controls (2·18 $\pm$ 0·34). The similarity of the initial phosphatase levels in normal and nephrectomised rabbits, despite the raised plasma Mg of the latter, suggested that the changes in Mg after limb ischaemia would be insufficient to account for the rise in phosphatase activity. This was further confirmed by the addition of Mg^{2+} to normal rabbit plasma where a 10-fold rise in the plasma Mg level only increased the alkaline phosphatase activity 22 per cent and had no effect on the acid phosphatase activity.

TABLE V.—*Effect of a 4-Hour Period of Bilateral Hind Limb Ischaemia on the Alkaline Phosphatase Activity of the Plasma of the Nephrectomised Rabbit.*

Rabbit no.	Initial level.	Phosphatase activity (units/100 ml. plasma) Time (hr.) after period of ischaemia. 0·5.	1.	2.	3.
174	7·0	8·4D	—	—	—
175	3·8	—	4·3D	—	—
2	10·0	—	9·4	8·9D	—
3	11·1	12·4	—	—	—
4	6·9	7·0	—	4·9D	—
6	5·0	5·2	—	—	3·9D
8	5·7	—	6·2	—	24·0D†
10	4·8	5·1	—	6·5	—
16	4·2	—	3·6	—	2·8
17	4·9	4·7	—	5·0D	—
Mean	6·3	7·1	5·9	6·3	3·4
S.E.*	0·24	0·31	0·37	0·37	0·54

D = Death due to limb ischaemia.
† = Specimen haemolysed (excluded).
* = Standard error calculated from " residual " mean square in analysis of variance.

Effect of Nephrectomy on the Changes after Limb Ischaemia.

Following the suggestion (Smith *et al.*, 1954, 1955) that the increase in plasma phosphatase was derived from the kidney the experiments were repeated on rabbits nephrectomised some 20 hr. previously. While this operation did not alter the clinical picture produced by this period of limb ischaemia it did affect the changes previously found in the alkaline phosphatase activity of the plasma (Table V). Except where definite haemolysis occurred, as in the terminal sample from Rabbit 8, the rise in activity after limb ischaemia was largely abolished. There was, however, still a definite ($P < 0.01$) increase in the plasma acid phosphatase after limb ischaemia in these nephrectomised rabbits (Table VI).

Effect of Limb Ischaemia on Liver and Kidney Phosphatase.

Determination of the alkaline and acid phosphatase activities of the liver and kidney in normal rabbits and of the liver in nephrectomised ones showed no

TABLE VI.—*Effect of a 4-Hour Period of Bilateral Hind Limb Ischaemia on the Acid Phosphatase Activity of the Plasma of the Nephrectomised Rabbit.*

Rabbit no.	Initial level.	Phosphatase activity (units/100 ml. plasma) Time (hr.) after period of ischaemia. 0·5.	1.	2.	3.
174	12·3	9·9D	—	—	—
175	4·1	—	2·7D	—	—
2	5·3	—	5·1	5·8D	—
4	3·1	5·5	—	7·8D	—
6	4·8	5·1	—	—	7·4D
8	4·6	—	7·2	—	16·4D†
10	7·0	10·0	—	8·9	—
16	4·8	—	7·8	—	12·5
17	11·2	13·5	—	18·3D	—
Mean	6·5	8·8	5·7	10·2	9·9
S.E.*	0·54	0·72	0·81	0·81	1·14

D = Death due to limb ischaemia.
† = Specimen haemolysed (excluded).
* = Standard error calculated from " residual " mean square in analysis of variance.

significant difference between those from the controls and those after limb ischaemia. For instance, the kidney alkaline phosphatase activity in 7 control rabbits averaged 6560 ± 2380 units (see " Methods ") per 100 g. wet wt. while the average value in 9 experimental rabbits was 6040 ± 2180 units.

The histochemical test for alkaline phosphatase proved more sensitive probably because of its ability to reveal changes confined to groups of cells which would not affect the biochemical assay. Kidneys from rabbits subjected to limb ischaemia studied by this method showed loss of activity in the tubules (Fig. 3 and 4) and occasional phosphatase-positive casts. The extent of the loss varied considerably. In some, large areas of the cortex were affected while in others the loss occurred in small patches. No changes were detected in the liver after limb ischaemia.

DISCUSSION.

These experiments show that the increase in plasma phosphatase activity seen by Smith *et al.* (1955) in fatal anthrax in the guinea-pig also occurs after a fatal period of limb ischaemia in the rabbit. The rise in the alkaline phosphatase activity of the rabbit plasma was not so striking as in anthrax. This might be due to the difference in species, but apart from this the survival time after limb ischaemia was much shorter. On the other hand the increase in plasma acid phosphatase after limb ischaemia was much greater than in untreated anthrax.

Smith *et al.* (1955) and Ross (1955) consider the increased plasma alkaline phosphatase activity represents a movement of enzyme from the tissues, in particular the kidney, into the blood stream. It is difficult to argue from alteration in enzyme activity to a change in concentration of enzyme protein and before this inference is accepted the activation of existing enzyme must be excluded. Alkaline phosphatase can be activated by Mg^{2+} and certain amino acids and dipeptides (Jenner and Kay, 1931 ; Thoai, Roche and Roger, 1947 ; Akamatsu and Aso, 1954). The changes in plasma Mg concentration after limb ischaemia proved insufficient to account for the effect. The influence of the raised plasma amino

acid level after limb ischaemia (Hoar and Haist, 1944) has not been excluded directly. However, the absence of increased plasma alkaline phosphatase activity in the nephrectomised rabbit after limb ischaemia where the changes in amino acid level will be at least as great, seems to rule out this possibility. The effect of nephrectomy strongly suggests that the increased plasma activity is due to an excess of enzyme derived from the kidney. The histochemical results, resembling those reported by McManus (1950) in human cases of " crush syndrome " and shock, by Berg and Levinson (1952) in dogs given *Clostridium perfringens* toxin and by Ross (1955) in anthrax in guinea-pigs, are in agreement with this. The occurrence of phosphatase-positive casts is particularly important since it shows that the enzyme can leave the cells of the renal tubules without loss of activity. The failure of biochemical assay to show any fall in the alkaline phosphatase activity of the kidney must be explained. The advantages of the histochemical test have been given above. The kidney is so rich in alkaline phosphatase that only a small fraction, probably less than 5 per cent, need be lost to the blood stream to produce the observed rise in plasma activity. Such a loss would be invisible against the large variation in normal concentration. The shortness of the experiments may be another factor for Migone, Fiaccadori and Scarpioni (1953) found that much longer periods (14 hr.) of limb ischaemia were required to reduce the kidney alkaline phosphatase activity in a similar biochemical assay.

The kidney is not so intimately concerned in the elevation of the plasma acid phosphatase level. There is no evidence that this represents an activation of existing enzyme. While the greater effect of Seitz over Millipore filters probably reflects adsorption by the former, the removal of part of the excess by filtration suggests its association with circulating cell débris such as " sludged " blood (Knisely, Eliot and Bloch, 1945) or degenerating lymphocytes, both types of cell being rich in acid phosphatase (King, Wood and Delory, 1945 ; Haight and Rossiter 1950). Since these phenomena are common to many forms of injury the rise in plasma acid phosphatase may be considered a feature of the general response to injury.

Regarding the mechanism of the alkaline phosphatase changes it is relevant that Migone and Fiaccadori (1953) and Gowing and Dexter (personal communication) have found a similar loss of activity in the renal cortex and the formation of phosphatase-positive casts after transient renal ischaemia. A fall in renal blood flow can be confidently assumed in the present experiments from the work of Trueta, Barclay, Daniel, Franklin and Pritchard (1947) and others. Since the renal circulation is reduced by many types of injury the changes in the alkaline

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EXPLANATION OF PLATES.

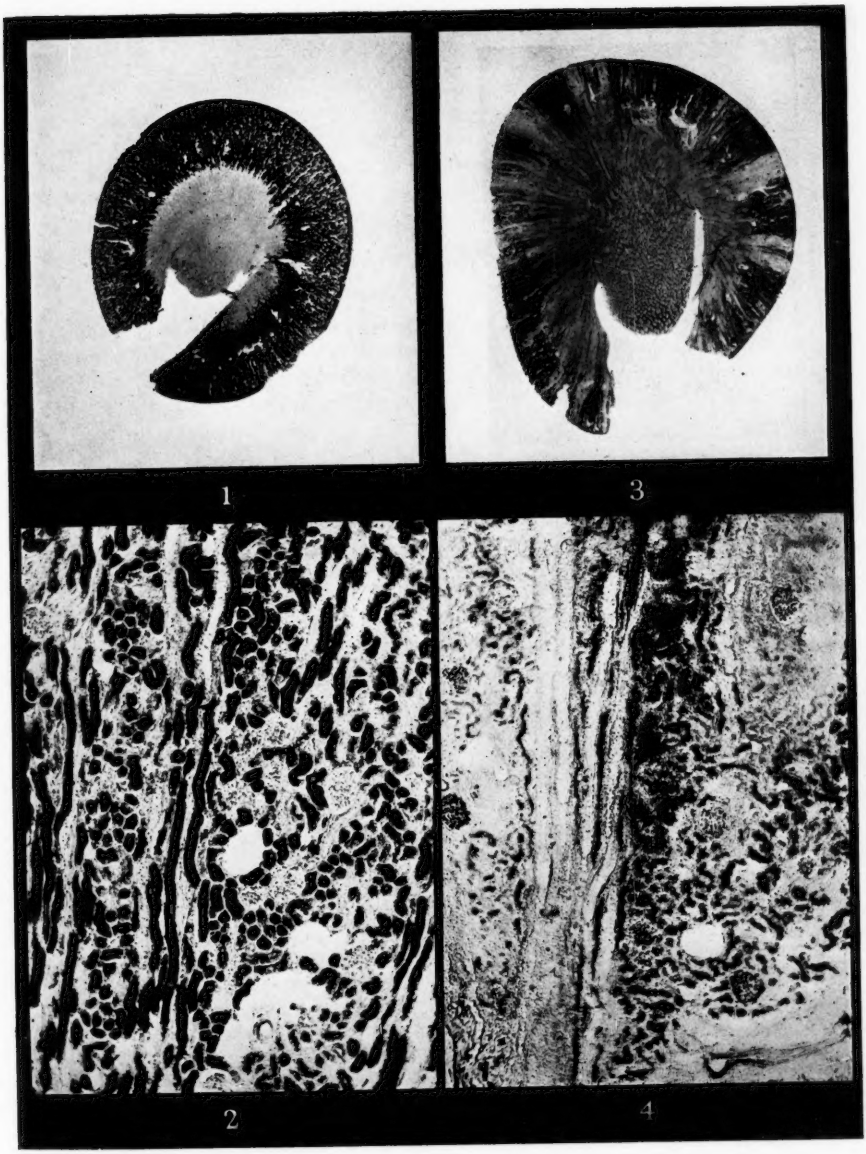
The alkaline phosphatase distribution in the renal cortex of the rabbit. Frozen sections (10μ) from neutral 10 per cent buffered formalin-fixed material treated according to the method of Pearse (1953) without counterstaining.

FIG. 1.—Kidney (× 2) from control rabbit. Alkaline phosphatase reaction confined to the cortex.

FIG. 2.—Renal cortex from the same rabbit at a higher magnification (× 50) showing the alkaline phosphatase reaction of the renal tubules.

FIG. 3.—Kidney (× 2) from a rabbit killed 4 hr. after a 4-hr. period of bilateral hind-limb ischaemia showing the patchy loss of the alkaline phosphatase reaction in the cortex.

FIG. 4.—Renal cortex from the same rabbit at a higher magnification (× 50) showing loss of the alkaline phosphatase reaction and dilatation of the renal tubules.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phosphatase activity of the plasma and kidney should also be considered part of the general effect of injury. Whether these plasma changes would be of any help in the clinical assessment of renal damage after injury awaits further study. There is also the question whether these changes play any part in the production of the response of the body to injury. Smith *et al.* (1955) tentatively suggested that they might be concerned in the changes in carbohydrate metabolism. It must however be remembered that the increased plasma levels are demonstrated by tests carried out *in vitro* at 37°. Injury reduces the body temperature (Stoner, 1954) and as the Q_{10} of the plasma alkaline phosphatase was found to be 2·05 and that of the acid phosphatase 2·07 the changes in activity *in vivo* may be quite small. The full significance of these results cannot be appreciated until the physiological function of these enzymes is known.

SUMMARY.

Limb ischaemia in the rabbit was found to cause an increase in the alkaline and acid phosphomonoesterase (phosphatase) activities of the plasma and a decrease in the alkaline phosphatase activity of the renal cortex. Nephrectomy prevented the rise in the plasma alkaline phosphatase activity without greatly affecting the change in acid phosphatase activity. It is thought that the excess plasma alkaline phosphatase is mainly derived from the kidney following anoxic renal damage. The main source of the excess acid phosphatase is not known. It is possibly associated with the increased destruction of the cellular elements of the blood following injury.

These changes are considered in relation to the findings after other forms of injury, notably anthrax, and it is concluded that they represent part of the general response to injury.

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THE CHEMICAL BASIS OF THE VIRULENCE OF *BACILLUS ANTHRACIS*. III: THE RÔLE OF THE TERMINAL BACTERAEMIA IN DEATH OF GUINEA-PIGS FROM ANTHRAX.

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Bacillus anthracis and its products have been obtained from infected guinea-pigs in sufficient quantity for chemical extraction (Smith, Keppie and Stanley, 1953*a*). It has been suggested that the examination of this type of *in vivo*-produced material might be the best way of identifying the chemical compounds and processes responsible for the virulence of this and other pathogenic organisms (Smith, Keppie and Stanley, 1953*b*). An immediate approach to the identification of the chemical basis of the invasiveness of the organism was made. Agressins were easily demonstrated in extracts of the organisms (Smith *et al.*, 1953*b*) and extracellularly in the body fluids of the infected guinea-pigs (Keppie, Smith and Harris-Smith, 1953). These two crude products are being chemically fractionated for agressins (Smith and Zwartouw, 1954; Smith, Zwartouw and Gallop, 1954).

Unfortunately, in so far as the cause of death in anthrax is concerned, the literature (see Smith *et al.*, 1953*b*) had little to offer other than speculation. Neither the general nature of the lethal effect on the host nor even the products or processes of the organism mainly responsible for the characteristic symptoms of the disease were known. No study of the chemical basis of this aspect of pathogenicity was therefore possible until the nature of the fatal syndrome was established and the substance causing it recognised. Work leading to the solution of this problem, already briefly reported (Smith and Keppie, 1954; Smith, Keppie, Ross and Stanley, 1954), is described in more detail here and in two subsequent papers.

Up to the present, no lethal endo- or exotoxin has been found in *in vitro* cultures of *B. anthracis* (Eurich and Hewlett, 1930; Sobernheim, 1931; King and Stein, 1950), and our first attempt to demonstrate a lethal factor in the products of the organism growing *in vivo* was unsuccessful. Large quantities of a mixture of five parts thoracic exudate and one part plasma from guinea-pigs dying of anthrax were not lethal when injected intraperitoneally into guinea-pigs. The result was the same when bacterial extracts were injected equivalent to an amount of bacteria twice that estimated to be present in a guinea-pig when it dies of anthrax (Keppie *et al.*, 1953). These preparations, although non-lethal, did produce the mild oedematous skin reaction already associated with such material (Sobernheim 1931; Cromartie, Watson, Bloom and Heckly, 1947; Watson, Cromartie, Bloom, Heckly, McGhee and Weissman, 1947). It was pointed out that this lack of gross toxicity and the fact that very large numbers of *B. anthracis* grew in the blood of guinea-pigs before they died meant that death was due to harmful effects which were weak relative to the number of organisms producing them.

Such effects already suggested in the literature are that the massive bacteraemia might block the capillaries of the host, produce a fatal anoxaemia or a deficiency of essential nutrients. To assess the importance of such hypotheses it was essential to know whether the bacteraemia had to reach its possible maximum before death from anthrax followed. It is generally agreed that a pronounced bacteraemia is a usual feature in death from anthrax in many species, and we have found it invariably so in our work with guinea-pigs. However, a few reports state that partly immune guinea-pigs (Sterne 1953, private communication) and some animals of other species (Bloom, McGhee, Cromartie and Watson, 1947; Stockman, 1911) die of anthrax with only a slight bacteraemia. Such reports were the exception to the general rule and were in the main incidental to other studies. A thorough investigation of the significance of the final bacteraemia in the death of guinea-pigs seemed necessary.

This paper is mainly concerned with the bacteraemia, but observations have been made on the significance of obvious changes in the blood of guinea-pigs dying of anthrax (Keppie *et al.*, 1953) and on the validity of two previous hypotheses (Bloom *et al.*, 1947; de Moulin, 1936) on the cause of death. Opportunity has also been taken to analyse the distribution of organisms in the tissues of the host at different stages in the final bacteraemia.

EXPERIMENTAL.

The Relationship between the Degree of Bacteraemia and Death from Anthrax.

The plan was to use therapeutic measures calculated to terminate the final bacteraemia abruptly at known progressive stages in its development. The subsequent fate of the guinea-pigs would indicate the earliest stage at which irreparable damage to the host had occurred.

The infection.—The strain of *B. anthracis* (N.P.) and the breed of guinea-pigs (700 g. $\pm$ 50) used were the same as in previous work (Smith *et al.*, 1953*a*), but here an intradermal injection (2×10^3 spores) was used. The animals died between the 3rd and 5th days after injection.

The final bacteraemia.—In the 12 hr. preceding death, the number of organisms in the blood rose from approximately 3×10^5 chains per ml. to 1×10^9 chains per ml. The progress of this final bacteraemia could be followed conveniently by microscopic examination of blood films from the ear. One flat loopful (area enclosed by loop 2 sq. mm.) of blood was spread over 1 sq. cm. and stained with methylene blue. Examination of 50–100 fields (magnification $\times$ 600) gave the average number of bacterial chains per field. In 15 comparisons of this method of counting with haemocytometer counts of the organisms, the relationship with the absolute count was obtained (1 chain per field = $1{\cdot}5 \times 10^7$ per ml. of blood) and the error of maximum scatter was within $\pm$ 50 per cent. After the degree of bacteraemia had been determined in this manner the survival time of the guinea-pig subsequent to the making of the blood film was noted. In Table I, the results given in the 2 left-hand columns show that the degree of bacteraemia is closely related to the period of survival; each survival time given is the average of observations on 10–15 animals in 6 experiments.

Termination of the bacteraemia: subsequent fate of the guinea-pigs.—A rapid method for terminating the bacteraemia was essential, and a number of therapeutic agents were tested for this purpose. Hyper-immune anthrax antiserum prepared in the horse (30 ml.), chlortetracycline (25 mg.), oxytetracycline (20 mg.) and chloramphenicol (100 mg.), failed to stop the rapidly developing bacteraemia. Penicillin was toxic for guinea-pigs at a dosage which was adequately bacteriostatic. Streptomycin, however, proved very effective and was non-toxic for guinea-pigs. The dosage used was an initial injection of 80 mg. (half given subcutaneously and half intraperitoneally), followed 6 hr. later by a further 40 mg. subcutaneously. Thereafter daily doses of 25 mg. dihydrostreptomycin cerate were given

TABLE I.—*The Relationship between the Degrees of Anthrax Bacteraemia in Guinea-pigs and the Time to Death. The Results of Streptomycin Treatment.*

Infected guinea-pigs.				Streptomycin-treated guinea-pigs.†		
Organisms in blood.		Hours until death.			Hours until death.	
No. per field.*	No. per ml. $\times 10^{-6}$ ($\pm$ 50%).	Average.‡	Standard deviation.	Number dead. / Number treated.	Average.	Standard deviation.
1/80	0·2	12·7	0·6	0/2	—	—
1/40	0·4	11·5	0·7	0/6	—	—
1/20	0·8	9·5	1·2	2/12	—	—
1/10	1·5	8·5	1·7	1/12	—	—
1/5	3·0	8·1	1·3	11/20	—	—
1/2	7·5	6·9	1·0	36/37	43	15
1	15	6·1	0·8	19/19	39	12
2	30	4·9	1·1	16/16	36	12
4	60	4·4	0·7	9/9	24	8
8	120	3·6	1·0	14/14	17	6
16	240	2·4	0·5	12/12	10	4
32	480	1·5	0·5	7/7	6	2
64	960	Death	—	—	—	—

* Films prepared as described in the text and stained with methylene blue, magnification × 600. Numbers refer to chains.

† See text for details of streptomycin treatment.

‡ Average of 10–15 animals in 6 experiments.

intramuscularly. The antibiotic rapidly terminated the infection. Multiplication of the organisms had ceased within 1–1½ hr., by which time they were disintegrating; after 5–7 hr. no bacilli remained in the blood. This applied even in animals treated approximately 4 hr. before they would otherwise have died. At times later than this, only 50–75 per cent of the animals could be freed from infection. The fate of animals treated with antibiotic at selected stages of the developing bacteraemia was noted, together with the time of death, if this occurred. Throughout the experiments, detailed observations were made only on animals freed from active infection.

The results of six experiments are summarised in the right-hand columns of Table I. Removal of the infection with streptomycin saved the guinea-pigs, provided the bacteraemia had not increased beyond *ca.* 3×10^6 chains/ml. of blood. All guinea-pigs with a bacteraemia greater than this critical value died although they were free from active infection. The time interval (6–72 hr.) between antibiotic treatment and death was much longer than that which would have elapsed had the bacteraemia not been stopped; it decreased regularly as the level of bacteraemia at which the animals were treated increased.

Observations on Two Recorded Hypotheses on the Cause of Death.

The study of the bacteraemia and the effective use of streptomycin to control it provided an opportunity to investigate previous hypotheses on the cause of death.

Interference with calcium metabolism.

Bloom *et al.* (1947) compared the symptoms of anthrax in rabbits (lassitude, drowsiness and excessive response to stimuli) with those of calcium deficiency, and noted the work of Weinstein (1938) who protected mice against anthrax by

injections of parathyroid extract. They showed that injections of calcium gluconate and laevulinate would to some extent protect rabbits against anthrax. The calcium salts and the parathyroid extract of Weinstein were given from the time of challenge onwards; their effect may have been on the initial invasion rather than on the killing power of the organism. Govaerts (1951) and Renaux (1952) noted the adverse effect on the virulence of *B. anthracis* when calcium salts were present in cultures *in vitro*.

In our work with guinea-pigs, no excessive response to stimuli was apparent. Administration of large quantities of calcium borogluconate (1 g. every 12 hr.) and parathyroid extract (0·5 mg.) separately or together, did not save life; furthermore it did not prolong the survival time of guinea-pigs (4 for each treatment) given streptomycin at a time immediately beyond the critical level of bacteraemia which predetermined the death of the animals 1–3 days later. The results of these experiments suggest that any action of the calcium or the parathyroid injections of Bloom *et al.* (1947) and Weinstein (1938) was not directed against the killing mechanism of the organism.

An attack on the central nervous system.

de Moulin (1936) reported that lesions occurred in the central nervous system of guinea-pigs infected with *B. anthracis*. He attributed the symptoms of anthrax and the death of the host to damage of this system. Our colleague Dr. Joan M. Ross has examined the histopathology of the tissues of guinea-pigs dying of untreated anthrax, and of those in which active infection had been stopped by streptomycin injection after the critical point in the bacteraemia. She could not detect the changes in the central nervous system described by de Moulin.

The Significance of the More Obvious Changes in the Blood of Guinea-pigs Dying of Anthrax.

Following earlier work (Sobernheim, 1931; Bloom *et al.*, 1947), Keppie *et al.* (1953) noted abnormalities in the blood of guinea-pigs dying from untreated anthrax. It was suggested that these abnormalities might have some bearing on the cause of death. Therefore a study was made of the R.B.C. agglutination, the slight haemolysis, the increased red-cell fragility, and the delayed clotting which occurs, with a view to deciding whether such abnormalities were present in the blood at the critical point of the bacteraemia or at the death of guinea-pigs freed from infection by streptomycin treatment 1–3 days previously. The general conclusion from this work was that such changes in the blood only occurred very late in the untreated disease and were not of primary importance. A summary of the results is given below. Observations were made on individual samples of heart blood from at least five animals.

Haemolysis.—The blood of guinea-pigs dead from untreated anthrax was 10–20 per cent haemolysed. This is a late phenomenon. Blood from animals approximately 6 and 1½ hr. before death from untreated anthrax was 1 per cent and 1–3 per cent haemolysed respectively. Animals dead after the streptomycin treatment described above had blood which was less than 1 per cent haemolysed.

Red cell fragility.—In the usual R.B.C. fragility test the concentration of NaCl solution in which haemolysis first occurs with cells from normal guinea-pigs is 0·40–0·45 per cent. The same concentration was necessary with the R.B.C.

from untreated animals approximately 6 hr. before death. This also applied to cells from animals treated with streptomycin at a time beyond which their lives could be saved. In fact, the only slight change in sensitivity was observed with cells from animals dead of untreated anthrax; here an NaCl concentration of 0·45–0·52 per cent gave first evidence of haemolysis.

Red cell agglutination.—The R.B.C. of guinea-pigs which had died from untreated anthrax were distorted and agglutinated in large masses. Agglutination occurs very late, commencing about 3 hr. before death. No agglutination had occurred in guinea-pigs about 6 hr. before death from untreated anthrax, or in those animals dying 1–3 days after removal of the infection with antibiotic.

Increased clotting time.

The fact that the blood of animals infected with *B. anthracis* clots poorly is well known (Bloom *et al.*, 1947) and this was readily confirmed in guinea-pigs. Blood (2 ml.) obtained by cardiac puncture was expelled immediately into a small tube at 37° in a water bath. The time for clotting was taken from the moment the blood first entered the syringe. Sixteen samples of normal blood clotted in 4–8 min. Samples of blood from 10 guinea-pigs dying from untreated anthrax clotted in 60, 24, 18, 10, 23, 6, 37, 14, 7 and 10 min.; some of the clots were incomplete. Samples of blood from 5 guinea-pigs dying 1–3 days after removal of the infection with streptomycin clotted in 13, 14, 17, 15 and 14 min., which is slightly slower than for normal blood.

Watson *et al.* (1947) found their crude "inflammatory factor" from rabbit oedema fluid had an anti-coagulant activity. We have been unable to find any pronounced anti-coagulant activity in the crude intra- and extracellular products of *B. anthracis* growing *in vivo* (Smith *et al.*, 1953*a*, *b*) or in purified fractions from them (Smith and Zwartouw, 1954; Smith *et al.*, 1954). The recalcification test of Foster (1941) was used, but citrated guinea-pig plasma was substituted for the sheep plasma. In a test in which heparin (0·0006 per cent) was active the following materials were inactive: dialysed plasma exudate (4 per cent), as well as the purified polyglutamic acid (1 per cent) and the highly active aggressin fraction (1 per cent) from it; ballotini bacterial extract (4 per cent); ammonium carbonate bacterial extract (4 per cent) and the following fractions from it, diffusate (1 per cent), purified polyglutamic acid (1 per cent), protein fractions (1 per cent), and purified polysaccharide (1 per cent).

The Distribution of B. anthracis *in the Tissues of Guiuea-pigs during the Final Bacteraemia.*

In these experiments the object was to determine if any particular tissue or organ harboured organisms in such selectively high numbers as to suggest a site for toxic action.

The organisms in the blood and major organs of the body were counted in tissue samples from groups of five guinea-pigs infected as described above and representative of the following three stages of the bacteraemia: pre-critical point when the blood contained 0·8–3 $\times$ 10^6 chains per ml.; post-critical point when the blood contained 4–15 $\times$ 10^6 chains per ml.; and at death. Suspensions for haemocytometer counts were prepared from spleen, liver, kidney, lung, skin and muscle by grinding the tissue with sand and water. Table II shows the relative

distribution of anthrax bacilli in these tissues. The numbers found in the muscle, the skin lesion, and the oedema of the primary lesion which extended into the peritoneal cavity were too low to be of any significance and are omitted from Table II. The total number in the guinea-pigs at death agrees with that obtained previously (Keppie *et al.*, 1953) for guinea-pigs infected by a different route. The most striking fact about the distribution of organisms is that at the critical point in the bacteraemia, the bacilli are concentrated in the spleen which contains over half the total number of organisms in the host; at death most of the organisms are in the blood stream.

TABLE II.—*Distribution of* B. anthracis *in the Tissues of Guinea-pigs* (700 *g.*) *during the Final Bacteraemia.*

Stage of bacteraemia at which samples were taken.*		Blood (Est. 40 ml.).	Spleen.	Lung.	Liver.	Kidney.	Total.
Pre-critical point (approx. 9 hr. from death)	1	1·0	4·5	0·9	0·8	0·1	7·3
	2	0·4	3·6	0·6	0·3	0·1	5·0
	3	0·4	2·2	0·2	0·1	0·1	3·0
	4	1·1	4·3	1·0	1·4	0·2	8·0
	5	1·4	2·8	0·9	0·3	Pr. nil	5·4
Post-critical point (approx. 6 hr. from death)	1	3·1	9·7	5·5	0·2	0·4	18·9
	2	1·7	10	1·6	0·1	1·2	14·6
	3	3·3	14	2·5	2·3	0·2	22·3
	4	1·7	13	5·2	2·9	0·2	23·0
	5	4·6	15	0·1	1·0	0·1	20·8
At death	1	440	110	25	7	13	595
	2	200	70	20	45	3	338
	3	260	40	8	4	13	325
	4	360	64	6	37	2	469
	5	440	96	37	44	6	623

* See text for details of these stages in the bacteraemia. Figures quoted are the total number ($\times 10^{-8}$) of bacterial chains (4–8 bacilli) in each tissue as determined by haemocytometer counts.

DISCUSSION.

In guinea-pigs a bacteraemia is intimately associated with death from anthrax, but in fact the death of the guinea-pig is determined when the bacterial invasion was still only about 1/300th of its possible maximum. This is experimental proof in a species which normally dies with a massive bacteraemia of a situation which was suspected on more limited observation in other species (Bloom *et al.*, 1947; Stockman, 1911). In small-scale experiments, we have been able to confirm the observation of Sterne (1953) that partially immune guinea-pigs may die after infection with *B. anthracis* with a mild degree of bacteraemia comparable to that existing at the critical point described above. There is a remarkably constant relationship between the increasing degree of bacteraemia and the decreasing period of survival in untreated guinea-pigs. This fact coupled with the quite large variation in the time to death after infection indicates that the animals are equally susceptible to the final killing stage of the disease but vary more in their ability to combat the initial infection. The relationship between the degree of bacteraemia and the extent of pathological damage is emphasised by the behaviour of animals treated with streptomycin. Any delay in chemo-

therapy subsequent to the critical point in the bacteraemia is reflected in a proportionally diminished survival time.

These experiments render untenable the hypotheses that death from anthrax is due to overwhelming numbers of organisms blocking the capillaries of the host or producing a deficiency in it of O_2 or essential nutrients (*e.g.*, glucose). Experiments reported here also shed doubt on the hypotheses of Bloom *et al.* (1947) and of de Moulin (1937) on the cause of death from anthrax. Furthermore, the more obvious changes in the blood of dying animals noted in previous work (Sobernheim, 1931 ; Bloom *et al.*, 1947 ; Keppie *et al.*, 1953) are terminal effects in untreated anthrax and largely disappear in those guinea-pigs dying of anthrax but free from infection as a result of streptomycin treatment. Hence a renewed search was made for the nature of the fatal syndrome, and in view of the relatively low level of bacteraemia which sealed the fate of a guinea-pig, for a toxic product of the organism which would produce this syndrome. Two subsequent papers describe the results of these investigations.

An analysis of the distribution of organisms in the host during the final bacteraemia had some bearing on the problem. It showed that at the critical point the spleen appeared to be fulfilling its function as part of the reticulo-endothelial system in removing large numbers of bacteria from the blood during the earlier period of active proliferation and spread. At this stage of the bacteraemia over half the total organisms were concentrated in the spleen. However, the figures for guinea-pigs at death showed that the spleen was unable to cope with the bacteraemia and soon the blood contained the majority of the organisms. The death of the host accompanies this final overflow. It is obvious, therefore, that any search for harmful effects or products of *B. anthracis in vivo* should pay particular attention to the blood and to the spleen.

SUMMARY.

A bacteraemia is intimately associated with death from anthrax in the guinea-pig. The fate of the animal is determined at a time when the bacterial proliferation in the blood is still only about 1/300th of the maximum obtaining at death. This finding renders untenable a number of hypotheses on the cause of death from anthrax which depend on the presence of a massive bacteraemia.

Evidence is presented against hypotheses of Bloom *et al.* (1947) and de Moulin (1936) on the cause of death from anthrax. Several abnormalities in the blood which were noted in previous work have been shown to be secondary side-effects.

An analysis of the distribution of organisms in guinea-pigs in the final phase of anthrax has focussed attention on the blood and spleen as possible sites for the production of harmful products.

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THE CHEMICAL BASIS OF THE VIRULENCE OF *BACILLUS ANTHRACIS*. IV: SECONDARY SHOCK AS THE MAJOR FACTOR IN DEATH OF GUINEA-PIGS FROM ANTHRAX.

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THE work described in the previous paper (Keppie, Smith and Harris-Smith, 1955) led to renewed search for a toxic substance produced by *Bacillus anthracis in vivo*. An insight into the probable nature of this factor was provided by the identification of the basis for the pathological syndrome which led to death. This was achieved by analyses of the blood, histological examinations and clinical observations on infected guinea-pigs.

The phase of the disease selected for these observations was the final 10 hours of the infection. The major pathological changes were expected to occur during this time because studies of the bacteraemia (Keppie *et al.*, 1955) had shown that the fate of infected animals was not decided until some 8 hours before death. Previous workers (Singer, 1927; Caifaleanu, Combiesco and Stamatesco, 1930; Bloom, McGhee, Cromartie and Watson, 1947) made observations throughout the duration of the disease, commencing at the time of initial infection. Their inconclusive results may be due in part at least to their having paid insufficient attention to this all-important final phase of the disease.

This paper describes the data which accumulated from numerous observations on infected guinea-pigs and how they fitted together to establish that "secondary shock" plays a major rôle in the death of guinea-pigs from anthrax.

In this paper, the term "secondary shock" (Cappell, 1951; Best and Taylor, 1950; Sodeman, 1950) means the pathological state involving circulatory failure which can be broadly summarised as "the general response of the body to injury" (Green and Stoner, 1954). Although the syndrome cannot be defined precisely, it is recognisable by the composite physiological disturbances which comprise it. Characteristic changes occur in the circulation, in the blood chemistry and in the distribution of the body fluids (Mellanby, 1954). The adjective "secondary" was chosen in preference to a more specific term such as haemorrhagic, traumatic, oligaemic burn, post-operative etc., because the nature of the injury in anthrax was incompletely understood.

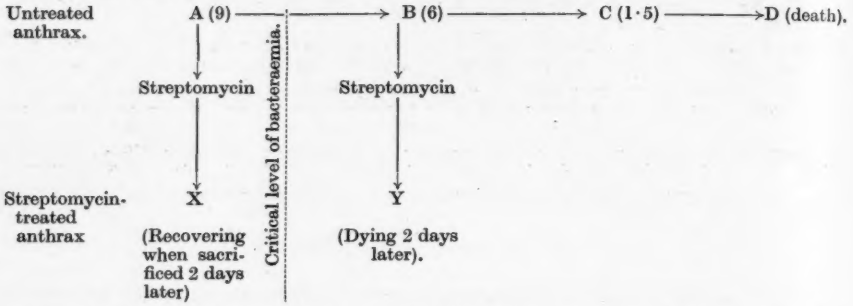
METHODS.

The breed of guinea-pig and the strain (N.P.) of *B. anthracis* were the same as those used in previous work (Smith, Keppie and Stanley, 1953*a*, *b*; Keppie *et al.*, 1955).

The stages of the disease studied.

Guinea-pigs (700 $\pm$ 50 g.) were infected intradermally with 2×10^3 *B. anthracis* spores and the bacteraemia was followed during the final 10 hr. of the disease as described in the previous paper (Keppie *et al.*, 1955).

Diagram Showing the Stages of Anthrax in Guinea-pigs which were Studied.



Stages of the final bacteraemia were determined by bacterial counts of the blood as described by Keppie *et al.* (1955). Figures in parenthesis are the average time (hr.) to death.

Observations were made at the following successive stages of the bacteraemia; they have been assigned letters, which will be used for the sake of brevity in presenting the tabular material. The Figure represents diagrammatically the following stages:

A: immediately before the critical point of the bacteraemia when the blood had 0·2–1·0 $\times$ 10^6 chains per ml. and when, in the absence of streptomycin treatment, death would ensue in approximately 9 hr.

B: shortly after the critical point, when there were 4–30 $\times$ 10^6 chains per ml., and when death would occur in approximately 6 hr.

C: at an advanced stage when the blood contained 2·5–6 $\times$ 10^8 chains per ml. and death would occur in about 1½ hr.

D: a few observations were made on animals immediately after death had occurred, or in the case of dynamic observations (*e.g.*, blood pressure) within ½ hr. of death.

Supplementary studies were made on the following guinea-pigs after removing the infection by treatment with streptomycin as described in the previous paper (Keppie *et al.*, 1955):

X: guinea-pigs treated with streptomycin at stage A above and observed or sacrificed for sampling 2 days later when recovering from the disease.

Y: guinea-pigs treated with streptomycin at stage B above and observed or sampled approximately 2 days later when they were dying free from active infection.

Experimental Procedures.

All observations and blood analyses were carried out on 5–20 animals. Quantitative results are shown as the mean (M) and standard deviation (S.D.) of the individual results. These observations were compared with those on a similar number of control animals. For comparison with the guinea-pigs of categories X and Y described above, control animals received the same dose of streptomycin as the experimental guinea-pigs.

All blood samples were taken by cardiac puncture under light ether anaesthesia. Analysis of whole blood samples from infected guinea-pigs was corrected for the *B. anthracis* organisms present. This was done by combining haemocytometer bacterial counts of the experimental sample with the appropriate analysis of known suspensions of *B. anthracis*. The organisms in these suspensions had been separated in a pure form from guinea-pig body fluids by a method of Smith *et al.* (1953*a*). Prior to plasma analysis the R.B.C. were removed by centrifuging at 500 r.p.m. for 10 min. followed by 2000 r.p.m. for 20 min.; samples were then sterilised by centrifuging at 8000 r.p.m. for 10 min. to remove most of the organisms, and filtering through "Millipore" filters (Lovell Chemical Co., Massachusetts). Filtration

was carried out in conditions where evaporation was negligible. The filters did not absorb materials from solution, and they left normal plasma unaltered in composition. However, all samples of plasma from normal animals were centrifuged and filtered for comparison with the infected samples.

Blood volume.—The blood volume of guinea-pigs was determined by a modification of the method described by Chaplin (1954) using R.B.C. labelled with ^{32}P. It was desirable to use homologous labelled R.B.C. for the reason discussed later.

A sample of blood (2 ml.) was collected for labelling from normal animals and each infected guinea-pig on the day prior to the onset of the final bacteraemia. Two μC of ^{32}P were added to each sample of blood and the mixture allowed to stand overnight at room temperature. Next day the blood was rotated at 37° for 1 hr. and the activated cells were separated from the plasma by centrifugation and washed thrice in 3 per cent plasma-saline. Immediately before they were required the cells were re-suspended to 2 ml. in the plasma-saline. Using local anaesthesia, 1 ml. of blood was removed from the jugular vein of the appropriate animal and replaced by 1 ml. of the suspension of labelled cells. After 15 min. heart blood was collected. The apparent blood volume of normal and infected guinea-pigs was obtained by comparing the radioactivity of the final heart blood with that of a 1/30 dilution of the original sample of labelled cells in normal guinea-pig blood. Concentrated nitric acid (8 ml.) was used to ash the blood samples (2 ml.), and their radio-activity was measured in the Veall liquid counter (Veall, 1948).

Blood pressure.—Using local anaesthesia the carotid artery of infected and normal guinea-pigs was cannulated with a polythene tube and a connection made to a mercury manometer through heparinised saline. The mean value of the pressure during the first 3 min. was determined.

Bleeding volume.—This was taken as the maximum volume of blood obtainable from uncomplicated cardiac puncture.

Haematocrit.—Heparinised blood was centrifuged (2000 r.p.m.) for ½ hr. in Wintrobe tubes.

Haemoglobin determination.—By the method of Clegg and King (1942).

Whole blood and plasma N.—By the Kjeldahl method. The whole blood value was corrected for the bacteria present, and the plasma value for a slight haemolysis (0–3 per cent) that had occurred. A haemolysis value of 1 per cent meant that a haemoglobin colour intensity equal to that of the plasma could be produced by diluting 1 part of whole normal blood with 99 parts of water, and that approximately 25 mg. of red blood cell protein was present in 100 ml. of plasma.

Plasma N.P.N.—The total nitrogen in the supernatant after precipitating a sample with trichloracetic acid (8 per cent).

Plasma protein N.—The difference between the total N and N.P.N.

Plasma glucose.—By the method of Hagedorn and Jensen (1923).

Body temperature.—The rectal temperature taken with a mercury thermometer after 3 min. *in situ.*

Sludged blood.—The flow of blood in the conjunctival vessels was observed by direct microscopical examination as described by Knisely, Bloch, Eliot and Warner (1947).

Phosphorus analysis.—By the method of Fiske and SubbaRow (1925). Total acid-soluble P and P as inorganic phosphate was determined after precipitation with trichloracetic acid (8 per cent w/v) for 10 min. at 0°. Blood lipoid phosphorus was determined by the method of Randles and Knudson (1922).

Electrolyte analysis.—Heparinised blood was collected and centrifuged under oil.

Chloride—by the method of King and Bain (1951). *Bicarbonate*—by the method of van Slyke (1922). A slight degree of haemolysis in some samples masked the phenol red indicator. This was therefore replaced by a mixture of bromothymol blue (0·2 ml.) and thymol blue (0·1 ml.). Plasma from control animals was treated similarly. *Sodium*—by the method of King (1951). *Potassium and magnesium*—we are grateful to Mr. L. C. Thomas and his colleagues of the Chemical Defence Experimental Establishment, Porton, Wilts. for these analyses (Table V) carried out by emission spectroscopy. *Calcium*—by the method of Peters and van Slyke (1932).

Plasma urea.—By the method of Harrison (1943) using prior aeration as described by van Slyke and Cullen (1916).

Plasma phosphatase analyses.—Alkaline, acid, and formolised acid phosphatase determinations were made by the methods of King (1951).

RESULTS.

Observations and Blood Analyses Relating to Secondary Shock in Guinea-pigs Dying of Anthrax.

The observations on, and blood analyses of guinea-pigs in the terminal phases of anthrax are described below under headings associated with secondary shock.

Clinical appearance.

Prior to the critical stage of the bacteraemia, infected guinea-pigs show no recognisable symptoms, but subsequently, the animals become increasingly weaker until death occurs quietly and almost imperceptibly. Marked loss of muscle tone is evident which eventually causes the animal to sink to the floor with legs extended. Breathing is characterised by forced inspiratory movement and it becomes increasingly shallow and slow.

Loss of blood volume.

An important feature of secondary shock is a reduced circulating blood volume (Sodeman, 1950 ; Lovatt Evans, 1952). Table I indicates clearly that there is a definite decrease in blood volume in the terminal phase of anthrax infection, and that the decrease is of the order of 25 per cent approximately 1½ hr. before death. The values, however, can only be taken as a reasonable estimate of blood volume for the following reason.

Determination of haemoglobin on the blood taken from the jugular vein before injecting the labelled cells and on the final heart blood showed that a fall in haemoglobin content occurred when the radioactive cells were circulating. This loss of haemoglobin indicated the disappearance of red blood cells from the circulation during the crucial 15 min. period. There is the possibility that radioactive cells were removed in this manner, which would affect the accuracy of the method for blood volume. The loss of haemoglobin occurred in control animals to the extent of 2–10 per cent, with a tendency to be larger if heterologous blood was used. In infected animals, use of homologous labelled blood gave losses of 2–10 per cent in the majority of cases. Use of heterologous labelled blood tended to give higher haemoglobin losses, sometimes as high as 20 per cent. Only results from experiments in which the haemoglobin loss was less than 10 per cent have been recorded in Table I.

TABLE I.—*Blood Volume, Bleeding Volume and Blood Pressure of Guinea-pigs (700 ± 50 g.) during the Final Bacteraemia of Anthrax.*

Nature of test.		Control animals.	Guinea-pigs at progressive stages in the final bacteraemia.* A.	B.	C.	D.
Blood volume (ml.)	M	39	38	30	29	24†
	S.D.	2·9	2·9	4·7	2·1	2·9
Bleeding volume (ml.)	M	20	17	13	7	—
	S.D.	2	2	1·5	1·5	—
Blood pressure (mm. Hg.)	M	100	84	56	39	23
	S.D.	7	10	9	12	8

* For details of these stages see text. Five to 20 guinea-pigs in each group.

† The blood volumes of these guinea-pigs are perhaps less reliable than the remainder, since the labelled red blood cells circulated only 5–10 min. before the animal died.

Reduced bleeding volume.

A number of workers (*e.g.*, Tabor and Rosenthal, 1947) have used the maximum amount of blood obtainable by bleeding to exhaustion as a measure of the circulating blood volume ; it is much reduced in secondary shock. Table I shows that a large and progressive reduction of bleeding volume occurs in the terminal phases of untreated anthrax.

Lowered blood pressure.

A fall in blood pressure is a criterion of shock (Sodeman, 1950 ; Lovatt Evans, 1952). The results given in Table I show the dramatic fall in blood pressure in the last 8 hr. of untreated anthrax.

Haemoconcentration.

In secondary shock loss of plasma from the circulation leads to haemoconcentration, whereas haemorrhage leads to haemodilution (Sodeman, 1950 ; Magee and Spector, 1951–52). The results of haemoglobin, haematocrit and blood and plasma N determinations on different batches of animals shown in Table II agree

TABLE II.—*Evidence for Haemoconcentration in Guinea-pigs during the Terminal Phases of Anthrax.*

Blood analysis.†		Guinea-pigs at progressive stages in the final bacteraemia.* Controls.	A.	B.	C.	Streptomycin-treated controls.	Guinea-pigs after removing the infection with streptomycin.* X.	Y.
Haematocrit	M	41	44	49	48	44	46	50
	S.D.	3·1	3·0	5·5	2·6	4·8	6·2	6·4
Haemoglobin concentration (per cent w/v)	M	15·6	16·8	17·9	18·9	—	—	—
	S.D.	1·4	1·0	1·7	1·3	—	—	—
Total blood N. (g. per 100 ml.)	M	2·97	3·02	3·20	3·03	2·94	3·07	3·13
	S.D.	0·10	0·18	0·15	0·09	0·09	0·19	0·13
Total plasma N. (g. per 100 ml.)	M	0·78	0·59	0·58	0·61	0·77	0·55	0·56
	S.D.	0·02	0·07	0·07	0·07	0·05	0·01	0·05

* For details of these stages see text.
† Each different analysis was done on separate batches of from 5 to 20 guinea-pigs.

in indicating a 10–20 per cent haemoconcentration in guinea-pigs dying of treated (Y) and untreated (C) anthrax, and also in those animals immediately beyond the critical point in the bacteraemia. There are indications that haemoconcentration is not so marked in guinea-pigs before the critical point in the bacteraemia (A), and in those saved by streptomycin treatment (X). The fact that haemoconcentration is not markedly greater at stage C in the bacteraemia than at stage B may be accounted for by haemorrhage in the terminal phases, and perhaps more by sludging of the R.B.C. (see later) which are consequently trapped in the tissues.

Presence of oedema and haemorrhage.

The loss of circulating blood volume occurring in secondary shock is due to haemorrhage, loss of plasma to the site of the burn or injury, or trapping and

pooling in the peripheral circulation (Sodeman, 1950; Grant, 1954; Courtice, 1954). In untreated guinea-pigs, haemorrhage and oedema occurred at the injection site on the flank, and also retroperitoneally around lymph nodes in the lumbar region. This macroscopic oedema had increased greatly in those guinea-pigs dying free from infection two days after administration of streptomycin.

The loss of plasma proteins to the tissues was adequately confirmed by plasma protein nitrogen analyses. Values for plasma protein N (mg. per 100 ml.) for stages A, B and C in the bacteraemia were 539 (S.D. 72), 519 (S.D. 78) and 517 (S.D. 45) respectively, compared with 745 (S.D. 21) for control animals. Similarly, streptomycin-treated animals X and Y had plasma protein N values (mg. per 100 ml.) of 480 (S.D. 41) and 404 (S.D. 69) respectively, compared with 731 (S.D. 57) for control animals.

Fall in body temperature.

In secondary shock there is decreased metabolism leading to diminished energy production, and one sign of this is a fall in body temperature (Stoner, Threlfall and Green, 1952). The average rectal temperature of guinea-pigs (20) dying from untreated anthrax fell from 37·2° to 31·1° during the final 6 hr. of the disease. Similar experiments on animals dying after streptomycin treatment (Y guinea-pigs) showed that the fall was 33·3°–26·6° in the final 10 hr. of life.

Interference in carbohydrate metabolism.

Secondary shock is accompanied by a serious interference in carbohydrate metabolism associated with the lowered metabolic rate and body temperature (Stoner *et al.*, 1952). A rise in plasma glucose levels occurs followed by a terminal drop. In infected guinea-pigs, plasma-glucose analyses revealed a reduction of glucose levels in the later stages of the disease. Figures (mg. per 100 ml. plasma) of 167 (S.D. 17), 189 (S.D. 24), 135 (S.D. 26) and 51 (S.D. 10) were obtained at stages A, B, C and D in the bacteraemia, compared with 158 (S.D. 8) obtained on control animals which, like the infected animals, had been fasting 24 hr. In another study our colleague D. W. Tempest has confirmed that a hyperglycaemia occurs earlier during the blood invasion; he obtained values of 212 (S.D. 4) in infected guinea-pigs approximately 12 hr. before death. In interpreting the results of glucose analyses on the plasma of animals infected with *B. anthracis*, the rise in glucose levels can be considered as indicative of pathological damage. The terminal drop in glucose levels must be in part due to the fact that this substance is used by the growing organisms in the rapidly developing bacteraemia, but the low glucose value at death is undoubtedly significant.

Sludged blood and prolonged clotting time.

The well known delay in the clotting of the blood of animals infected with *B. anthracis* (Bloom *et al.*, 1947) has been confirmed in our work with guinea-pigs (see previous paper). This abnormality in the blood, together with the *in vivo* sludging (Knisely, Eliot and Bloch, 1945) of the blood of infected guinea-pigs which we have observed 3–4 hr. from death, are also symptoms of secondary shock (Sodeman, 1950: Knisely *et al.*, 1947).

Phosphate metabolism.

The inorganic phosphate content of plasma rises in secondary shock (Magee and Spector, 1951–2). The total and organic phosphorus content of the R.B.C. also rises (Allison, Cole, Holmes and Root, 1947). The results of P analyses on the blood of animals dying of untreated and streptomycin-treated anthrax shown in Table III are identical with those found in secondary shock.

TABLE III.—*Phosphorus Content (mg. per 100 ml.) of Whole Blood and Plasma of Guinea-pigs Dying of Anthrax Compared with that of Normal Guinea-pigs and Guinea-pigs given Streptomycin.*

Type of analysis.		Controls.	Stages of the bacteraemia in untreated anthrax.* A.	B.	C.	Streptomycin-treated controls.	Guinea-pigs after removing the infection with streptomycin.* X.	Y.
Whole blood :								
Total P	M	33·9	40·0	41·5	43·3	34·6	40·2	47·2
	S.D.	1·2	3·1	2·3	2·4	1·8	5·4	1·8
Acid sol. P	M	27·0	32·1	33·6	34·1	27·9	32·1	38·7
	S.D.	1·1	2·0	2·2	2·3	1·4	4·2	1·0
Inorganic P	M	4·1	5·9	6·5	10·0	5·3	6·7	15·4
	S.D.	0·5	1·2	0·6	1·7	1·0	1·7	2·7
Lipoid P	M	7·0	8·6	8·8	9·2	7·5	9·0	10·0
	S.D.	0·6	0·5	0·7	0·8	0·3	0·6	0·7
Plasma :								
Total P	M	5·6	9·7	9·3	12·9	8·0	10·8	20·9
	S.D.	0·2	2·1	1·0	1·4	0·9	1·6	2·5
Acid sol. P	M	4·4	8·0	7·9	12·1	6·8	8·4	17·8
	S.D.	0·3	1·5	1·0	1·6	1·1	1·6	3·1
Inorganic P	M	4·6	7·9	8·0	11·9	5·8	8·2	17·6
	S.D.	0·4	1·4	1·2	1·4	1·2	1·4	3·3

* For details of these stages see text. 5–20 animals per group.

Disturbance in electrolyte balance.

A disturbance in electrolyte balance occurs in shock. The pH of the plasma falls, as does the content of Na and HCO_3. The plasma K and Mg content rise, but changes in Ca and Cl concentrations are somewhat inconsistent (Magee and Spector, 1951–2 ; Root, Allison, Cole, Holmes, Walcott and Gregerson, 1947). The results recorded in Table IV show that there is a large disturbance in electrolyte balance in the terminal phases of untreated anthrax. The changes in the concentrations of electrolytes are in agreement with those obtained in shock. Plasma Ca, Mg and K rose and Cl and HCO_3 levels decreased as the anthrax bacteraemia progressed. The pH of the plasma dropped at the end of the bacteraemia. The change in Na concentration was not consistent. At the early stages (A and B) in the bacteraemia the Na content was reduced. Application of a *t*-test to the analyses of the plasma at these stages and to those of plasma from control animals showed that this reduction was highly significant ($P = < 2$ per cent for analyses at stage A, < 1 per cent for analyses at stage B, and $< 0{\cdot}1$ per cent for a combin-

TABLE IV.—*Plasma Analyses for Chloride, Bicarbonate, Sodium, Potassium, Calcium and Magnesium on Guinea-pigs during the Final Stages of Untreated Anthrax.*

(Figures are mg. per 100 ml. plasma.)

Plasma analysis.		Controls.	Stages in the final bacteraemia of untreated anthrax.* A.	B.	C.
Cl	M	376	361	359	345
	S.D.	5	5	7	11
HCO_3	M	145	129	113	83
	S.D.	15	16	24	13
Na	M	324	310	313	332
	S.D.	8	14	8	19
K	M	15·7	15·5	16·2	26·4
	S.D.	2·5	1·8	2·4	3·9
Ca	M	13·0	12·3	15·2	21·8
	S.D.	0·5	0·5	2·2	7·5
Mg	M	2·6	2·1	2·5	5·7
	S.D.	1·1	0·5	0·5	1·4

* For details of these stages see text, 5–20 animals per group.

ation of both). At a later stage (C) of the bacteraemia no significant reduction of the Na content could be detected in numerous determinations ; in fact there was a tendency for it to rise slightly. The reason for this is not clear.

Evidence of acute renal failure.

Secondary shock leads to damage of kidney tissue and renal failure (for references see Green, Whitely and Eglin, 1951 ; Dible, 1953). The gradual development of uraemia was shown by analyses of plasma N.P.N. and urea (Table V) in guinea-pigs at all the various stages of anthrax described above. At 9 and 6 hr. before death from untreated anthrax (stages A and B) they showed little evidence of uraemia. Their plasma N.P.N. and urea values were only

TABLE V.—*N.P.N. and Urea, Analyses of the Plasma as Indication of Renal Failure in Guinea-pigs Dying of Treated and Untreated Anthrax.*

(Figures are mg. per 100 ml. plasma.)

Plasma analysis.		Controls.	Guinea-pigs at progressive stages in the final bacteraemia of untreated anthrax.* A.	B.	C.†	Streptomycin-treated controls.	Guinea-pigs after removing the infection with streptomycin.* X.	Y.†
N.P.N.	M	35·8	51·2	58·3	91·7	38·0	68·5	185
	S.D.	3·6	8·7	10·7	10·5	5·0	23·4	15
Urea	M	53·6	67·6	77·3	115	47·6	97·8	284
	S.D.	7·1	15·2	7·8	12	7·0	33·7	44

* For details of these stages see text, 5–20 animals per group.
† The urine of these animals contained much protein.

30–50 per cent above the normal values. At about 1½ hr. before death (stage C) the rise in these values had become significant.

Acute renal failure occurred in animals treated with streptomycin, but dying 2–3 days later although free from infection (stage Y). In these animals anuria developed. Streptomycin-treated control animals when allowed to drink at will produced 25–35 ml. of urine daily (10 animals). The infected guinea-pigs produced an average 6·5 ml. of urine (27 animals) on the day following the streptomycin injection ; this average fell to 3 ml. on the next day (14 animals). Daily subcutaneous or intraperitoneal injection of saline (30 ml.) produced no significant change in the volume of urine passed. Calcium (7·5 (S.D. 0·9)) and P as inorganic phosphate (17·6 (S.D. 3·3)) analyses (mg. per 100 ml.) on the plasma of type Y animals were also characteristic of renal failure. Animals which were saved by early treatment with streptomycin (type X), developed some degree of anuria but within a week or so could excrete normal quantities of urine. Fourteen animals of this type passed a daily average of 10, 9, 16, 22 and 30 ml. of urine on the 1st 2nd, 3rd, 6th and 12th day after the termination of the infection with streptomycin.

In animals dying after streptomycin treatment, the question arose whether the renal failure could seriously affect a guinea-pig in such a short time as 2–3 days. Our colleague J. Widdicombe tied off the ureters of 18 guinea-pigs (700 g.). These animals died in 2·5 (2), 3 (3), 3·5 (10), 4 (2) and 4·5 (1) days after the ureters were blocked ; the number dying in each time interval is given in brackets, and it will be noted that the average survival time after this operation is not dissimilar from that of type Y guinea-pigs. The plasma of these dying guinea-pigs contained protein N 720 (S.D. 41) ; N.P.N. 341 (S.D. 21) ; and urea 611 (S.D. 41) mg. per 100 ml.

The indication of renal failure in guinea-pigs infected with *B. anthracis* given by the plasma analysis led to a study of the histopathology of the kidneys of both treated and untreated animals. Our colleague Dr. Joan M. Ross describes this study in a following paper. In brief, tubular damage was found similar to that characterising a number of conditions (*e.g.*, " traumatic uraemia ", " crush syndrome ") which give rise to anuria, and have as a common feature a disturbance of the general circulation. The degree of damage at the various stages of the disease agreed with the plasma chemistry described above.

Plasma phosphatase analyses on guinea-pigs dying of anthrax ; the connection with kidney damage.

The high plasma inorganic phosphate content of infected guinea-pigs prompted a determination of plasma phosphatase levels. The alkaline phosphatase values in Table VI show that the content of this enzyme in plasma increased as the bacteraemia progressed and was extremely high approximately 1½ hr. before death. The connection of the high alkaline phosphatase figures with death and the parallelism with the evidence of renal failure was shown by analyses on the streptomycin-treated animals. Animals dying after streptomycin treatment (type Y) had high plasma alkaline phosphatase levels, whereas the plasma of those saved by the antibiotic had a relatively low enzyme content when sacrificed 2 days after treatment.

These exceptionally high alkaline phosphatase values merited further investigation. The liver was not apparently the source of the enzyme, for there was no

TABLE VI.—*Plasma Acid and Alkaline Phosphatase Estimations on Guinea-pigs Dying of Treated and Untreated Anthrax.*

(Figures are the phosphatase units of King (1951) per 100 ml. plasma.)

		Guinea-pigs at progressive stages in the final bacteraemia of untreated anthrax.*			Streptomycin-treated controls.	Guinea-pigs after removing the infection with streptomycin.*	
	Controls.	A.	B.	C.		X.	Y.
Plasma analysis :							
Alkaline phosphatase M	6·3	21	34	191	6·4	15	42
Alkaline phosphatase S.D.	2·4	4·4	13	40	2·3	3·7	12
Acid phosphatase M	5·3	6·6	6·0	7·6	10·9	7·5	20
Acid phosphatase S.D.	0·8	1·0	2·6	1·7	2·6	2·8	3·0
Formalised acid phosphatase M	3·1	4·0	4·1	4·0	5·8	3·4	17
Formalised acid phosphatase S.D.	0·6	0·8	0·4	0·6	1·1	0·9	3·0
Urine analysis :							
Alkaline phosphatase M	*Nil*	—	—	1756	—	—	—
Alkaline phosphatase S.D.	—	—	—	469	—	—	—

* For details of these stages see text.

evidence of obstructive jaundice or of gross histological damage, and tests on the various types of plasma for bilirubin (Haslewood and King, 1937), thymol turbidity (Maclagan, 1944) and gold number (Maclagan, 1946) showed no abnormality. No significant amount of alkaline phosphatase could be detected in extracts of *B. anthracis* grown *in vivo*, or in *in vitro*-grown culture filtrates. However, the high concentration found in the urine of infected guinea-pigs (see Table VI) suggested that the kidney might be a source of the enzyme. Subsequently Dr. Joan M. Ross confirmed that the kidneys of infected guinea-pigs showed marked loss of this enzyme.

High plasma levels of alkaline phosphatase and loss of this enzyme from the kidney have not been generally recognised as features of shock. A few authors (McManus, 1950; Berg, Levinson and Wang, 1951; Berg and Levinson, 1952) have noted a loss of alkaline phosphatase from the kidney in shock cases, but until now no plasma enzyme determinations have been carried out. Recently Dr. H. B. Stoner (private communication) has shown that ischaemic shock in rabbits results in a raised plasma alkaline phosphatase content. It is possible that some of the alkaline phosphatase of the plasma comes from tissues other than the kidney, which must, however, be one source of the enzyme.

The abnormally high formalised acid phosphatase content of the plasma of guinea-pigs dying two days after removing the infection with streptomycin is interesting. This was not investigated further, although it is not improbable that the kidney is also the source of this enzyme.

DISCUSSION.

A quantitative comparison of the results obtained in experimental anthrax in the guinea-pig with those obtained in fatal cases of secondary shock (for definition see introduction) shows that this pathological state occurs in the terminal phases of anthrax and is the major cause of death. In this comparison it will be apparent

that two types of death have been indicated by our results. The animals dying 7–8 hr. after the critical point in the bacteraemia of untreated anthrax are in a state of shock, whereas in those animals treated with streptomycin just after the critical point and dying two days later, renal failure which results from shock plays a large part. In untreated animals, the rapid and progressive fall in bleeding volume (60–70 per cent), in blood pressure, in body temperature and in plasma glucose parallels the situation in fatal shock as do the disturbances in phosphate, and other plasma electrolytes (Stoner *et al.*, 1952 ; Tabor and Rosenthal, 1947). The streptomycin-treated animals showed anuria, high levels of plasma urea, N.P.N. and inorganic phosphate, low plasma Ca, and the histological changes in the kidney which characterise the fatal acute renal failure found in cases of " crush syndrome ", " traumatic anuria ", etc. (Dible, 1953). The significance of these data in relation to the cause of death is emphasised by the corresponding more normal observations from animals (X) which recovered when streptomycin had been given prior to the critical point of the bacteraemia.

It is of interest to consider the disturbance which primarily leads to the development of the secondary shock. Oligaemia, commonly accepted as one of the most important factors in shock, clearly occurs in guinea-pigs dying of untreated anthrax. The bleeding volumes suggest that a gross reduction of circulating blood volume occurs, but estimates of this reduction by the labelled red cell method indicate that $1\frac{1}{2}$ hr. before death it is 25 per cent, and that probably a further decrease occurs in the interval before death. Experiments on normal guinea-pigs have shown that the deaths following the removal of 35, 45 and 55 per cent of the blood volume were respectively 30, 50 and 100 per cent of the animals. These figures agree with statements that a loss of blood volume greater than 30 per cent is dangerous (Grant, 1954 ; Scott, 1954). Our figures for infected guinea-pigs approach these levels, but we cannot be sure that they are reached at death. Oligaemia appears to be largely responsible for the fatal state of shock, but the possibility that other metabolic disturbances contribute to this state is not ruled out.

It seems that in shock, alkaline phosphatase is set free from the kidney and perhaps from other tissues. The effect of these phosphatases on the phosphorylated intermediate metabolites of carbohydrate utilisation might be worthy of investigation, in view of the extensive interference in carbohydrate metabolism which occurs in shock (Stoner *et al.*, 1952).

Anthrax in many species has long been recognised as producing oedema and haemorrhage (Sobernheim, 1931). This loss of fluid from the circulation, if sufficient, will inevitably result in secondary shock. The delayed clotting of the blood and the interference in carbohydrate metabolism involving primary hyperglycaemia and terminal hypoglycaemia which are regularly found in anthrax (Cromartie, Bloom and Watson 1947 ; Bloom *et al.*, 1947) are general features of secondary shock as is also the clinical picture of increasing weakness and collapse. In their study of the blood chemistry of rabbits infected with *B. anthracis*, Bloom *et al.* reported little relating to shock apart from noting terminal rise of plasma N.P.N. We believe that this was due to their study of the disease throughout its length, rather than in the last 8–12 hr., when the symptoms would mainly be apparent.

Clinical evidence suggests that shock may play a rôle in a number of other infections, in which the cause of death remains obscure, *e.g.*, cholera (Napier, 1951).

However, that this pathological state does occur in man and in experimental animals has not been demonstrated by detailed biochemical and physiological studies comparable to those carried out on the classical types of shock. A study of other diseases along these lines might be fruitful. Thus some of the symptoms of shock follow the injection of certain bacterial toxins, such as those of the gas gangrene group (Berg *et al.*, 1951 ; Berg and Levinson, 1952 ; Miles and Niven, 1950). Thomas (1954) has reviewed the work of Delaunay and others on the physiological disturbances produced by injection of the phosphorus-containing polysaccharide-protein-lipid complexes which constitute the endotoxins of the Gram-negative bacilli. They produce the clinical signs of shock, together with interference in carbohydrate metabolism, early disappearance of liver glycogen, and increase in blood inorganic phosphate, found in our work with guinea-pigs and in classical traumatic shock. Recently, Wattenberg, Elsberg, Wisseman and Smadel (1955) have produced fatal vascular disturbances in mice with rickettsial toxins.

The recognition of secondary shock as the pathological syndrome of anthrax started a search for an oedema-forming product of the pathogen responsible for the increased capillary permeability and the oligaemia of the host. This led to the demonstration of the specific lethal factor of *B. anthracis* already reported briefly (Smith and Keppie, 1954) and to be described in another paper.

These observations have been made solely with one strain (N.P.) of *B. anthracis* in the guinea-pig It is hoped to extend this work to test other strains and animal species.

SUMMARY.

A combination of studies on blood chemistry, physiology and histopathology have shown that a state of secondary shock occurs in anthrax in the guinea-pig and that it is the major cause of death. This finding is compatible with many previous observations on the pathology of anthrax. The identification of the pathological syndrome indicated the probable nature of the lethal factor produced by *B. anthracis*.

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ON THE HISTOPATHOLOGY OF EXPERIMENTAL ANTHRAX IN THE GUINEA-PIG.

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THIS paper is an annotation on material from guinea-pigs used in experimental work reported by Smith, Keppie, Stanley and Harris-Smith (1955). It describes in particular the histopathology of kidney from guinea-pigs at stages A, B and D (as described by Smith *et al.*) of the bacteraemia of untreated anthrax, together with material from guinea-pigs of their types X and Y from which the infection had been removed by treatment with streptomycin at stages A and B respectively.

Histological examinations have been carried out on only a small number of animals from each group, but there is a convincing uniformity in the findings in the various groups and the grading of damage. Formalin fixation was used whenever possible; experience had shown that tissues highly infected with *Bacillus anthracis*, in either the vegetative or spore form, were rendered sterile in the usual routine time of fixation. Material was collected into formol saline as a routine. Helly's fluid was used in some instances and formol saline chilled to 4° was found satisfactory for demonstration of alkaline phosphatase; provided the material was not left in fixative for more than 12 hours the activity of the enzyme was not greatly impaired.

All groups showed alterations in the kidney. In group A there was cloudy swelling affecting the tubules in the boundary zone with some loss of staining of the nuclei. The first part of the upper nephron showed some granular break-up of the mitochondria. In group B changes were more severe. The epithelial cells of the first convoluted tubule were swollen, the lumen of the tubule containing some cellular débris. Mitochondrial fragments were present in the débris. There was selective damage to the epithelium in the descending loop of Henle in the cortico-medullary region with degenerative changes in the cytoplasm and karyolysis in many tubules. Loss of desoxyribonucleic acid was demonstrable. In group Y the glomeruli were normal. There was cell degeneration in the proximal part of the nephron, especially in the descending loop of Henle at the boundary between the cortex and medulla (Fig. 1). In some nephrons this amounted to a definite cell necrosis (Fig. 2 and 3) and there were catarrhal desquamative changes. Many nuclei were undergoing karyolysis and loss of desoxyribonucleic acid was easily shown. There was no damage to the basement membrane of the tubules. The convoluted portion of the proximal nephron was also affected. The epithelium was swollen and the lumen reduced. Some tubules contained granular débris. Casts were present in the thin parts of Henle's tubule in the medulla and in some of the distal convoluted tubules in the cortex; these were usually hyaline and stained pink with eosin.

In the animals dying from bacteraemic anthrax (group D) the tubular changes described for group Y were present. They were slightly more severe and affected

the whole nephron although the tubules in the boundary zone were still the most severely damaged (Fig. 5). Casts were more frequent. The bacteraemia had reached a high level in these animals and thromboses in smaller vessels accompanied by large aggregates of bacilli were frequent (Fig. 6). In some cases glomeruli in the area supplied by these vessels were completely bloodless. Casts were present in the second convoluted tubules in the cortex (Fig. 4), in the thin loop of Henle in the medulla, and in some of the collecting tubules in the papilla (Fig. 4, 10 and 11).

Material from the kidney of the group D guinea-pigs was taken into chilled formol saline at 4° and fixed for 18 hr. before processing. Staining by Gomori's method (Pearse, 1953) showed the alkaline phosphatase in the brush borders of the epithelial cells of the proximal part of the nephron in control animals (Fig. 8 and 9). Kidneys of infected guinea-pigs when processed in this way showed that there was a general overall reduction in the amount of the enzyme in all animals (Fig. 7) and a complete absence in some of the proximal part of the nephron in the cortex and some reduction in the cortico-medullary zone. Casts in the second convoluted tubules in the cortex gave a positive phosphatase reaction and the casts in the collecting tubules were rich in alkaline phosphatase (Fig. 7 and 10).

DISCUSSION.

The changes observed in all tissues examined were those which might be expected in any acute infection. However, those found in the kidney were of special interest in so far as they helped in an understanding of the specific cause of death in anthrax. In this paper, therefore, attention has been given only to the examination of the kidney.

In the early stages of the bacteraemia, renal changes are those seen in any acute infectious disease. In later stages of the bacteraemia the changes are more severe and there is selective damage to the descending loop of Henle in the cortico-medullary region with degenerative changes in the cytoplasm and some karyolysis in many cells of the tubules. In animals treated with streptomycin at a stage too late to save life but in which the bacterial population has been eliminated, the tubular damage is most severe in the descending loop of Henle, amounting to cell necrosis in some nephrons. The changes are those of selective necrosis of the tubular epithelium. A term now regarded as obsolete, *viz.*, "lower nephron nephrosis" (Lucke, 1946) fully describes the picture (see Dible, 1953).

Smith *et al.* (1955) report an increased level of alkaline phosphatase in the plasma and urine of guinea-pigs dying from anthrax. The present study has shown corresponding decrease in kidney alkaline phosphatase. The diminution is of the same order as that described by McManus and Lowry (1948) in acute renal failure following trauma. The condition was later described by McManus (1950) in a general account of traumatic anuria (acute tubular necrosis) and was regarded as an index of renal tubular damage. However, no estimate of serum alkaline phosphatase seems to have been made in these patients. A similar situation exists over the experiments of Menton and Janouch (1946) with dogs receiving alloxan, and in those of Berg and Levinson (1952) on the action of *Clostridium welchii* toxin. The evidence on this point clearly requires strengthening. Thus Darmady (1954, personal communication) claims to have observed no increase in serum alkaline phosphatase in one classical case of crush syndrome where post-

mortem showed loss of this substance from the renal tubules. He also records similar experiences in cases of acute renal failure of other types. On the other hand, Stoner (1955) has produced evidence of increased plasma alkaline phosphatase in rabbits suffering from ischaemic shock and that the source of this was the kidney. In any event the mechanism of the removal of the alkaline phosphatase from the kidney is at present unknown.

The present study indicates the probability that kidney damage is brought about by the toxin or toxins produced from the growth of *B. anthracis in vivo*. Toxic action may be directly on the cells of the renal tubules, indirectly by some systemic action causing renal anoxia and so renal epithelial damage, or by a combination of these two factors. This is practically the same conclusion as that reached by Berg, Levinson and Wang (1951) when studying the effect of *Cl. welchii* toxin on the kidneys of dogs.

Recently (Editorial, 1954) it has been stated that the syndrome of acute renal failure initiated by renal anoxia (crush syndrome, traumatic anuria, etc.) is characterised not by necrosis of the renal epithelium alone but by the disruption of the basement membrane as well. In the same report it is stated that the syndrome due to the action of toxic substances directly on the renal tubule gives rise to necrosis of the epithelium only, but it is suggested that there may be an added systemic effect of the toxic substance producing renal anoxia. This may be the mechanism operating in death from *B. anthracis* infection.

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EXPLANATION OF PLATES.

FIG. 1.—Kidney, Y group; degenerative changes in descending loop of Henle in deep layer of cortex. H. and E.

FIG. 2.—Kidney, Y group; areas of necrosis in epithelium of descending loop of Henle. Boundary zone. H. and E.

FIG. 3.—Kidney, Y group; loss of nuclear staining in many of the epithelium cells of descending loop of Henle. Cellular débris is present in the lumen of the tubules. Boundary zone. H. and E.

FIG. 4.—Kidney, D group; deep layer of cortex, widespread degeneration of epithelium in first convoluted tubule and straight loop of Henle: casts in second part of nephron and collecting tubules. H. and E.

FIG. 5.—Kidney, D group; cortico-medullary region shows patchy necrosis of the epithelium of the straight loop of Henle, débris in lumen. H. and E.

FIG. 6.—Kidney, D group; shows dilated collecting tubules, thrombosis with numerous bacilli in blood vessels: empty glomerular capillaries. H. and E.

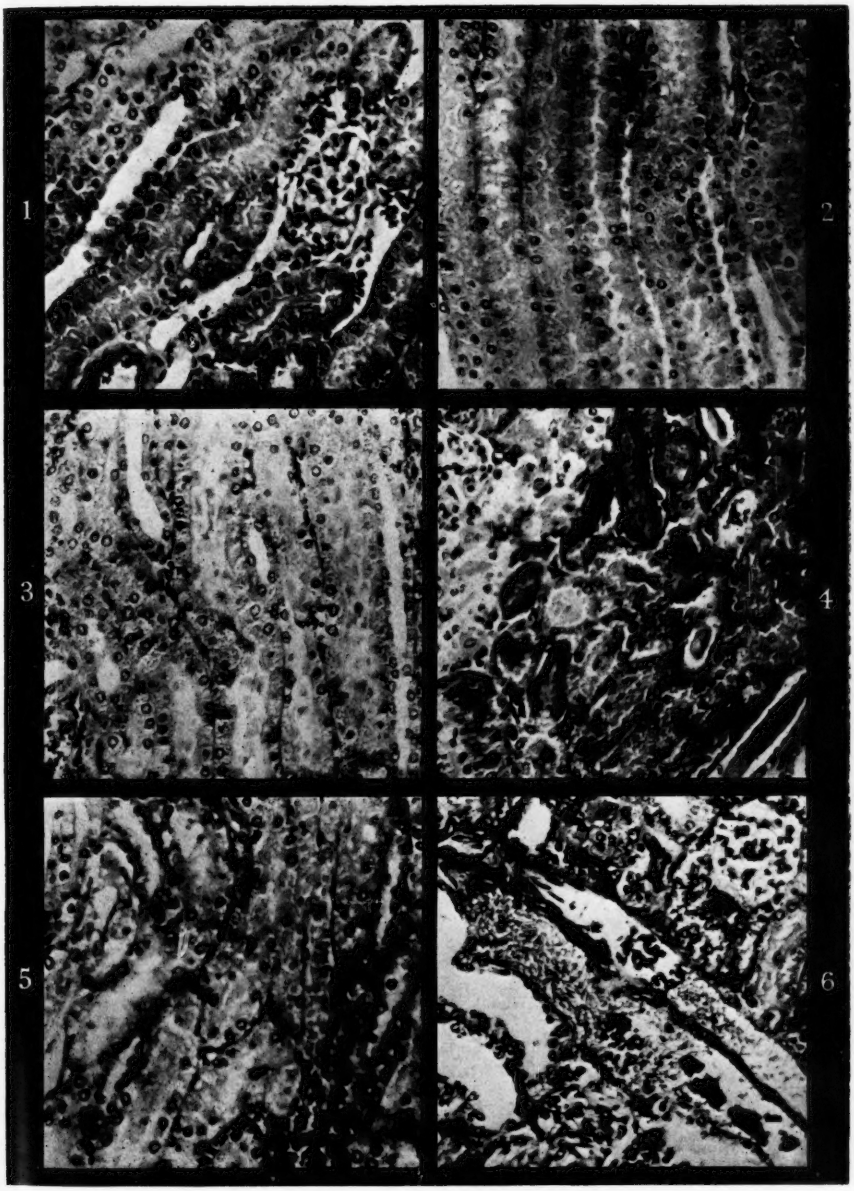
FIG. 7.—Kidney, D group. Low power view of section stained by Gomori's calcium cobalt method. Situation of alkaline phosphatase is shown in deep black. Note that blackening is scanty in the cortex, there is spread of blackening in the boundary zone and medulla and in the papilla. Some parts of the cortex show no blackening. × 10.

FIG. 8.—Kidney of normal guinea-pig. Low power view of cortex and medulla stained by Gomori's method. The second part of the nephron and the collecting tubules give no reaction. × 10.

FIG. 9.—Kidney of normal guinea-pig. Higher power of glomerulus and adjacent tubules in the cortex stained by same method. Glomerules and tubules of the second part of the nephron show no blackening which is confined to the brush border of the first convoluted tubule. × 100.

FIG. 10.—Kidney, D group. Casts in papilla stained by Gomori's calcium cobalt method to show alkaline phosphatase content of material in tubules. × 100.

FIG. 11.—Same kidney as Fig. 10. Casts in papilla stained H. and E. × 100.



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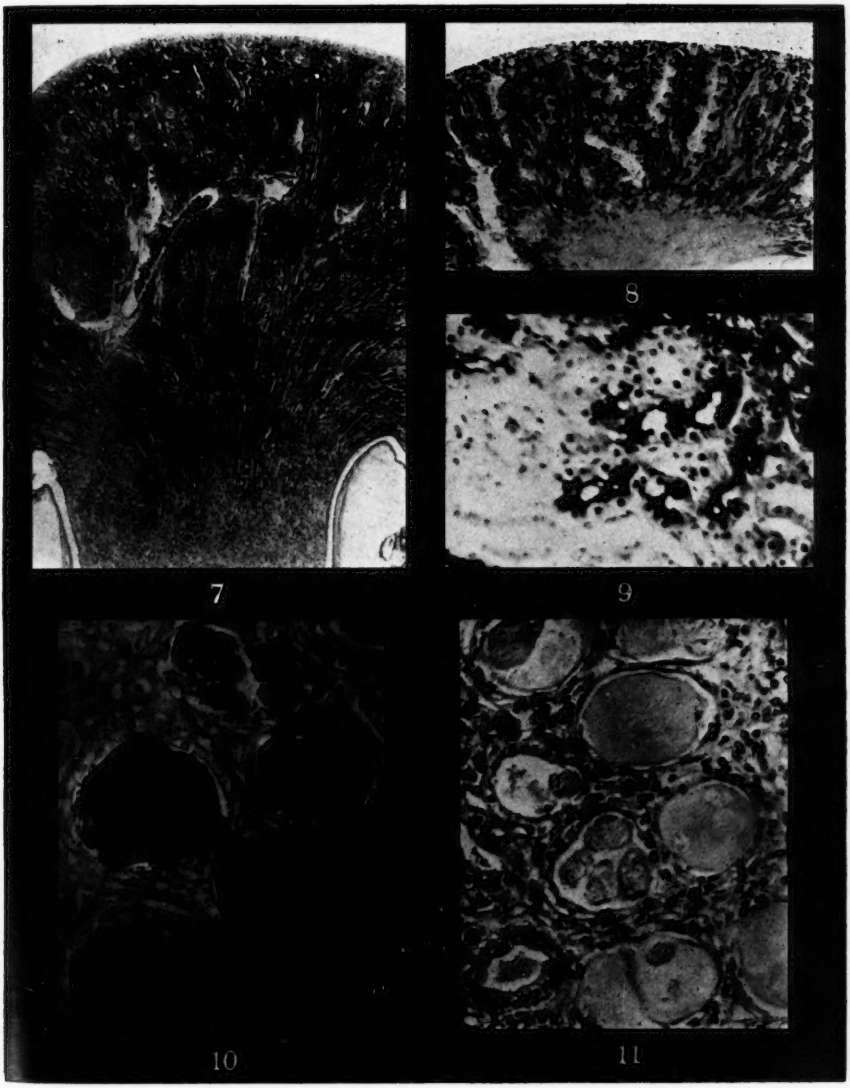

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SURVEY OF PAPERS.

WESTON HURST, SNOW and ROBERTS have studied the effects of mepacrine on equine encephalomyelitis in mice, guinea-pigs, rats, rabbits, chickens and monkeys, and have found that the marked activity in mice is not reproduced in the other species. From a study of the staining and biological properties of the basophilic particles found in the reticulo-endothelial system of mice they conclude that the particles contain an acid metabolite of mepacrine, and that the therapeutic effect is not dependent on the ability of a species to lay down particles (p. 215).

BIOZZI, BENACERRAF and HALPERN have found that the rate of phagocytosis of intravenously injected carbon in mice is reduced after one intravenous injection of a large dose of killed typhoid bacilli, but increased after subsequent doses. Subcutaneous injection of the bacilli stimulates phagocytosis from the start, with no initial depression. The endotoxin of *Salm. typhi* given intravenously has the same type of effect as the bacillary suspension. Cortisone interferes with the stimulation of phagocytic activity by these products (p. 226).

DOBSON, SANDERS and FLOREY have studied the effects of isoniazid, streptomycin or a combination of both on tuberculous lesions in rabbits using the ear chamber technique or systemic infection. Resolution of non-caseous lesions could be achieved by the drugs, alone or in combination, but even in these circumstances prolonged treatment did not eradicate the infection (p. 236).

WYNN WILLIAMS administered cortisone for 15 days to young guinea-pigs with the object of determining the mechanism of the exophthalmos so produced. Accurate skull measurements showed a retardation of bone growth compared with that in controls: the diminished size of the orbit would thus account for the protrusion of the growing eyeball (p. 245).

POOLE added fatty acids to citrated human plasma and by this shortened the coagulation time on recalcification. A similar effect of fatty acids was seen on the clotting time of plasma treated with Russell's viper venom, and on the fibrinogen time in the thrombin generation test (p. 248).

CYMERMAN-CRAIG, RUBBO and BARBARA J. PIERSON have extended their previous work on the anti-tuberculous activity in primary aromatic amines to the study of 46 diphenyl compounds. The activity of these is shown to depend both on the degree of ionisation and on the lipoid/water coefficient: the optimum value for each of these properties is defined (p. 254).

CYMERMAN-CRAIG, RUBBO, LODER and BARBARA J. PIERSON, in view of previous studies showing that anti-tuberculous activity depends on molecular planar surface area, degree of ionisation and lipoid/water partition coefficient, have compared these properties with capacity to inhibit the growth of tubercle bacilli in 27 aromatic heterocyclic bases. The results confirm previous findings, and show that compounds fulfilling all three criteria have a high degree of activity (p. 261).

HUMPHREY has shown that in rabbits made leucopenic by nitrogen mustard the lesions of reversed passive Arthus reactions are greatly diminished since local polymorph infiltration and vascular damage, by adherence of these cells to the endothelium, are suppressed (p. 268).

HUMPHREY has used polymorph-antiserum to produce leucopenia in guinea-pigs and has shown that, as in rabbits, Arthus reactions are largely, though not exclusively, related to the local action of polymorph leucocytes (p. 283).

KAY has obtained phage receptor material from a strain of *Salm. typhi* by repeated extraction with water and tested its capacity both in the crude state and after purification procedures to inactivate several typhoid phages. By studying the reaction between phage receptor and a phage containing ^{32}P he showed that the latter, presumably contained in nucleic acid, is liberated during the process of inactivation although at a rate slower than that of inactivation itself (p. 290).

WILGRAM and HARTROFT describe degenerative changes in the heart and vessels produced rapidly in young and more slowly in adult rats by a choline-deficient high-fat diet. The initial change in the vessels is a deposition of fat in the intima, followed by the formation of a plaque, and underlying medial necrosis and calcification. Fatty change succeeded by necrosis also occurred in heart muscle. The effects were aggravated by also giving cholesterol, although there was no consequent rise in the serum cholesterol (p. 298).

STONER has found that limb ischaemia in the rabbit causes an increase in the alkaline and acid phosphatase activities of the plasma, and a decrease in alkaline phosphatase activity of the renal cortex. The rise in plasma alkaline phosphatase activity was prevented by nephrectomy, which did not greatly affect the acid phosphatase level. These results are discussed in relation to other injuries and the findings reported in the three succeeding papers (p. 306).

KEPPIE, SMITH and PATRICIA HARRIS-SMITH have studied the terminal bacteraemia in guinea-pigs suffering from anthrax. Terminating the infection in some animals with streptomycin, a task beyond chloramphenicol, oxytetracycline and chlortetracycline, they have shown that the death of a guinea-pig is determined when the bacterial invasion is only about 1/300 of its possible maximum. They conclude that a massive bacteraemia *per se* and a number of other hypotheses cannot be held to account for death from anthrax (p. 315).

SMITH, KEPPIE, STANLEY and PATRICIA HARRIS-SMITH have studied the blood chemistry, physiology and histo-pathology of guinea-pigs either dying from anthrax or in whom the infection was terminated by streptomycin. In their opinion the findings, including the falls in bleeding volume, blood pressure, body temperature, and in plasma glucose levels, and the disturbance in phosphate and other plasma electrolytes, suggest that secondary shock is the major cause of death in guinea-pigs dying from anthrax (p. 323).

JOAN ROSS has examined kidney from guinea-pigs studied in the preceding paper. She finds that in the early stages of the bacteraemia renal changes are those seen in any acute infectious disease. In later stages the changes are more severe and there is selective damage to the descending loop of Henle in the cortico-medullary region with degenerative changes in the cytoplasm and some karyolysis in many cells of the tubules. There was also a decrease in kidney alkaline phosphatase (p. 336).

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